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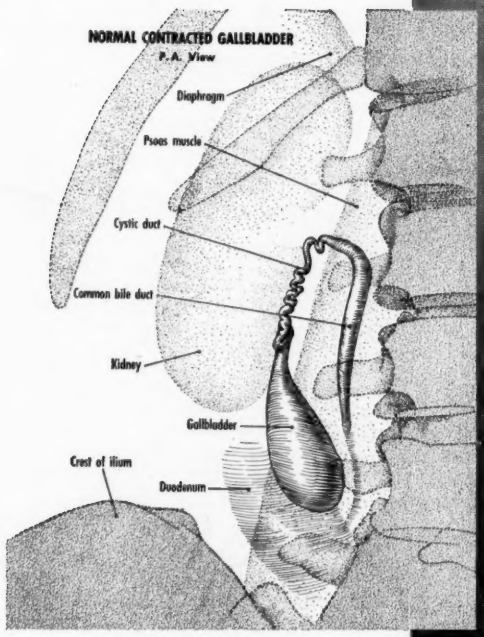

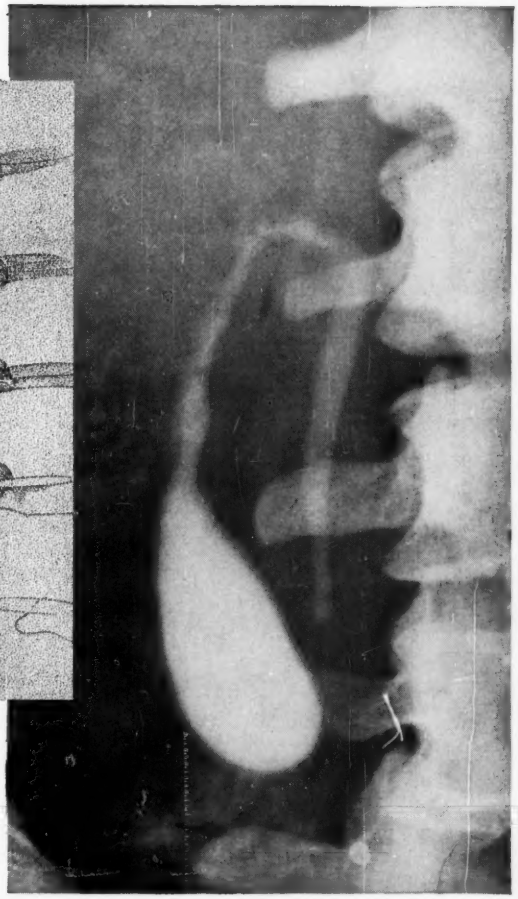

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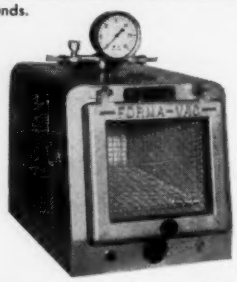

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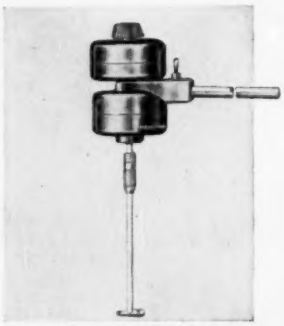

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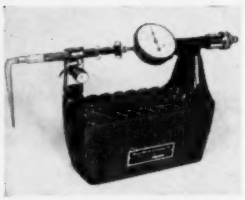

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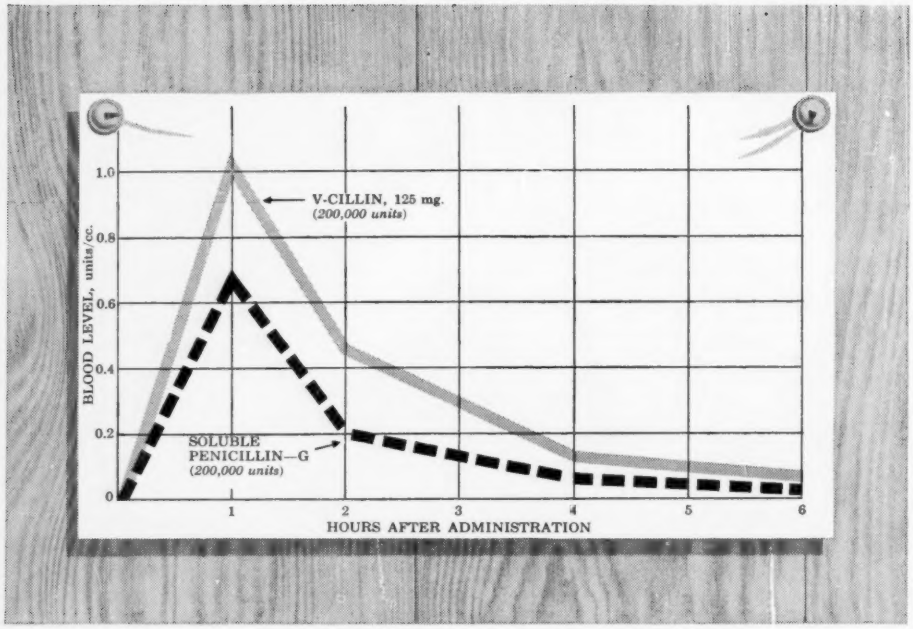

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RELATION OF CIRCULATING RED CELL VOLUME TO BODY DENSITY AND OBESITY[1]

By REX L. HUFF AND D. D. FELLER

(From the Radioisotope Unit and Medical Service, Seattle Veterans Administration Hospital, and the Department of Medicine, University of Washington School of Medicine, Seattle, Wash.)

(Submitted for publication June 27, 1955; accepted September 7, 1955)

The over-all metabolic activity of adipose tissue in man and other mammals has not been defined as carefully as that of other major organs and tissues. Despite recent reports (1, 2) showing that adipose tissue partakes in many biochemical reactions common to other tissues, widespread opinion holds that fat depots serve little purpose other than storage.

A primary index of metabolic activity in any organ is the blood flow through the organ. In this regard, some investigators favor the opinion that blood volume in the adult remains unaltered as body weight increases with fat deposition, and therefore blood volume and body weight correlate poorly in adults and should be well correlated with lean body weight (3, 4).

The study reported here was undertaken in order to obtain an estimate of the range of circulating red cell volume in men and women having differing degrees of obesity in an attempt to assess the relative importance of obesity tissue as an "organ" associated with a "fixed " quantity of blood.

METHODS

Circulating red cell volume

A modification of the technique of Sterling and Gray (5) was used for measuring circulating red cell volume. About 10 to 20 microcuries of chromium 51 in 0.01 to 0.2 ml. containing about 20 to 120 micrograms sodium chromate was injected into a sterile rubber-capped 15 ml. centrifuge tube. About 10 to 12 milliliters of the subject's heparinized blood was added to the solution and incubated for 45 minutes at room temperature. The incubated blood was spun at 1500 rpm for 5 minutes in an International centrifuge (size 2, model R). The supernatant was then withdrawn and the cell mass reconstituted with saline. The latter procedure was repeated twice. An aliquot of one milliliter, measured in a 1-milliliter calibrated syringe, was injected into a 100-milliliter volumetric flask for a standard, and the remainder was given to the recumbent subject by a single intravenous injection. The quantity given was estimated by reading from the calibration of the 10-milliliter syringe. Venous blood samples were drawn with a heparinized syringe at 5 and 10 minutes after injection, and two 4-milliliter aliquots of each sample were pipetted into vials, spun at 1000 rpm for 5 minutes, and analyzed in a conventional well-crystal detector. Efficiency of the well-crystal detector was constant for any volume up to 2.5 milliliters. Hematocrits were measured by centrifuging blood at 3000 rpm for one-half hour. Two-milliliter aliquots from the 100-milliliter volumetric flask were counted in a similar manner for calculation of the amount of radioactivity injected.

Circulating red cell volume was calculated as follows:

$$1.\quad \frac{\dfrac{(\text{Cts./min./ml. std.})(100)(\text{ml. injected})}{\text{Cts./min./ml. blood}}}{\text{Hct.}} = \text{Circulating red cell volume (CRCV)}$$

No correction was made for retained plasma between the packed cells.

Blood volume was calculated by the following formula:

$$2.\quad \frac{(\text{Cts./min./ml. std.})(100)(\text{ml. injected})}{\text{Cts./min./ml. blood}} = \text{Circulating blood volume}$$

and

3. Plasma volume by the difference of "2" and "1".

We believe, that with the exception of retained plasma in the packed cells, this method *measures* circulating red cell volume; plasma volume and blood volume are calculated values and not measured values. The former reservation is made because of the general knowledge of the possible difference in the total body hematocrit and the sampled hematocrit.

Body density

Weight of the subjects was determined with a standard physician's scale which had been calibrated with known volumes of water at a known temperature. Gross body volume was obtained from the volume of water displaced by the patient when he was immersed with the chest in full expiratory phase. Immersion was in an upright cylindrical galvanized iron tank. The tank was designed with metal rungs along the inside wall. The subject was instructed to forcibly exhale just prior to

[1] This work was supported in part by a grant from the Life Insurance Medical Research Fund.

submersion. The rungs aided the subject to attain and maintain submersion in a half sitting posture.

The water of the tank was connected through a tube from the bottom to a burette on the side. The tank was calibrated by recording burette readings with the addition of 2-liter aliquots of water. A calibration curve was thus obtained from which the volume represented between any two points on the burette could be read. Water temperature was usually maintained in the range of 34° to 37° C. The volume of an inanimate object could be measured with this system to an accuracy of ± 0.25 liters.

Residual lung volume was measured by the closed circuit method of Christie using the apparati and applying the formulae described by him (6). The subjects were seated in a fashion simulating their posture in the tank and instructed to forcibly exhale to the same expiratory point, before and after connection to the spirometer. Although this procedure did not measure "true residual air" and was subject to error on the part of subject participation, values obtained were considered well within the over-all accuracy required by the design of the experiment.

The mean values for residual lung volumes were found to be 2.54 liters for men and 2.03 liters for women with standard deviations of .08 liters and .05 liters, respectively.

Body density was then the quotient of the weight and the net body volume (immersion volume minus the residual lung volume). This measurement is density, or mass of body per unit volume, and can be converted to specific gravity by dividing by the density of water at

TABLE I

Normal men

Subject	Age (*Yrs.*)	Ht. (*M.*)	Wt. (*kg.*)	Lean wt.* (*kg.*)	Fat wt.* (*kg.*)	R. C. vol. (*L.*)	Plasma vol. (*L.*)	Hct. %	Blood vol. (*L.*)	Body vol. (*L.*)
1	32	1.81	73.9	47.0	26.9	1.99	2.67	42.7	4.66	71.75
2	21	1.73	67.6	43.9	23.7	2.06	2.02	50.5	4.08	65.46
3	28	1.88	78.8	47.8	31.0	1.73	2.58	40.2	4.31	76.79
4	38	1.70	97.8	48.7	49.1	2.66	2.99	47.2	5.64	97.12
5	26	1.73	64.4	55.1	9.3	1.58	1.80	46.8	3.38	60.06
6	48	1.70	67.5	50.6	16.9	2.34	2.52	48.2	4.86	64.11
7	32	1.79	74.0	38.8	35.2	1.72	2.03	45.9	3.74	73.10
8	27	1.78	94.5	57.8	36.7	2.60	2.99	46.5	5.59	92.06
9	32	1.85	83.4	52.0	31.4	2.00	2.60	43.6	4.60	80.98
10	31	1.82	71.0	54.0	17.0	2.17	2.81	41.5	4.98	67.35
11	38	1.82	100.4	57.2	43.2	2.16	2.63	45.1	4.79	98.42
12	48	1.84	97.5	60.8	36.7	3.06	2.56	54.6	5.62	94.71
13	24	1.71	80.5	46.4	34.1	2.18	2.74	44.4	4.92	78.87
14	37	1.74	100.9	42.3	58.6	2.54	3.07	45.0	5.61	101.56
15	34	1.71	59.0	49.0	10.0	1.86	2.00	48.2	3.85	55.30
16	54	1.67	109.5	67.9	41.6	2.50	2.53	49.6	5.03	106.55
17	30	1.79	92.9	60.6	32.3	2.39	2.71	46.9	5.10	89.88
18	45	1.80	112.5	78.1	34.4	2.72	3.07	47.0	5.79	107.99
19	36	1.66	127.7	65.4	62.3	3.26	2.99	52.3	6.25	126.45
20	24	1.88	69.8	57.9	11.9	2.53	3.11	44.8	5.64	65.41
21	26	1.83	73.6	62.6	11.0	2.16	2.51	46.0	4.67	68.69
22	30	1.69	59.3	56.3	3.0	1.90	2.47	43.4	4.37	54.37
23	29	1.78	81.5	57.1	24.4	1.95	2.46	45.3	4.31	78.15
24	60	1.70	92.5	51.8	40.7	2.96	3.19	48.9	6.05	90.71
25	34	1.88	62.4	52.4	10.0	1.97	2.51	44.1	4.48	58.39
26	29	1.72	69.0	50.6	18.4	1.78	2.24	44.3	4.02	65.80
27	30	1.90	74.4	59.8	14.6	2.40	2.56	48.3	4.96	70.07
28	31	1.78	82.2	53.2	29.0	2.12	2.72	43.7	4.84	79.59
29	28	1.84	77.3	63.4	13.9	2.28	2.64	46.4	4.92	72.61
30	28	1.79	110.3	54.7	55.6	2.80	3.05	47.8	5.85	110.18
31	32	1.78	69.0	62.1	6.9	2.00	2.49	44.6	4.49	63.88
32	27	1.68	61.4	58.3	3.1	2.44	2.53	49.2	4.97	56.90
33	22	1.78	64.8	42.1	22.7	2.00	2.51	44.4	4.51	62.70
34	20	1.84	75.3	67.8	7.5	2.37	3.12	43.9	5.39	69.65
35	21	1.83	75.3	65.5	9.8	2.08	3.01	40.9	5.09	70.08
36	29	1.77	71.6	61.6	10.0	2.33	2.77	45.7	5.10	66.72
37	34	1.84	83.9	56.4	27.5	2.08	2.42	46.3	4.50	80.84
38	30	1.70	69.9	50.5	19.4	2.20	2.37	48.0	4.57	66.59
39	28	1.74	83.8	72.5	11.3	2.30	2.83	44.9	5.13	78.07
40	32	1.84	81.0	66.0	15.0	1.62	2.55	39.0	4.17	76.13
41	16	1.86	86.9	79.4	7.5	2.44	2.99	45.0	5.43	79.08
42	26	1.93	84.6	74.7	9.9	2.51	3.49	43.0	6.00	78.63
Mean	32	1.78	81.3	63.88	17.40	2.26	2.66	45.8	4.91	77.90
S.D.	9	0.07	15.6	9.16	11.01	0.37	0.35	3.0	0.66	16.40

* These values were calculated from the Rathbun-Pace fat prediction equation (7).

TABLE II

Normal women

Subject	Age (*Yrs.*)	Ht. (*M.*)	Wt. (*kg.*)	Lean wt.* (*kg.*)	Fat wt.* (*kg.*)	R. C. vol. (*L.*)	Plasma vol. (*L.*)	Hct. %	Blood vol. (*L.*)	Body vol. (*L.*)
1	35	1.59	41.7	38.4	3.3	1.23	1.53	44.4	2.76	38.47
2	30	1.63	62.3	43.0	19.3	1.66	2.07	44.7	3.73	59.87
3	30	1.71	69.7	38.3	31.4	1.67	2.15	43.9	3.82	68.57
4	29	1.62	61.4	36.2	25.2	1.60	1.98	44.5	3.58	60.04
5	34	1.68	81.3	39.8	41.5	1.70	2.68	38.7	4.37	80.85
6	25	1.76	59.8	53.1	6.7	1.47	2.13	40.8	3.60	55.44
7	26	1.67	62.6	46.9	15.7	1.21	2.12	36.3	3.33	59.48
8	30	1.59	48.7	28.7	20.0	1.27	1.75	42.0	3.02	47.50
9	29	1.60	51.1	42.4	8.7	1.35	1.79	42.9	3.14	47.86
10	28	1.67	54.8	34.2	20.6	1.39	2.03	40.6	3.41	53.28
11	30	1.62	49.4	41.0	8.4	1.36	1.76	43.4	3.12	46.26
12	57	1.59	55.6	30.1	25.5	1.27	2.07	38.4	3.34	54.79
13	66	1.56	65.1	31.2	33.9	1.37	1.73	44.1	3.10	64.79
14	37	1.68	72.7	54.2	18.5	1.72	2.63	39.4	4.35	69.23
15	46	1.76	74.7	50.8	23.9	1.52	2.45	38.3	3.97	71.90
16	25	1.62	57.1	46.4	10.7	1.47	2.25	39.5	3.72	54.66
17	36	1.64	50.9	43.1	7.8	1.20	1.96	38.0	3.16	47.57
18	29	1.72	64.6	41.7	22.9	1.37	2.09	39.7	3.46	63.30
19	62	1.69	65.1	31.3	33.8	1.57	2.06	43.0	3.63	64.78
20	31	1.59	48.9	31.9	17.0	1.27	1.99	38.9	3.25	47.29
Mean	36	1.65	59.9	45.33	14.54	1.43	2.06	41.1	3.49	57.80
S.D.	11	0.06	9.8	6.69	7.08	0.17	0.28	2.6	0.41	10.21

* These values were obtained from the Rathbun-Pace fat prediction equation (7).

that temperature. Specific gravity assignment of the living human body is limited in general to a comparison of water at 37° C.

The percentages of lean and fat weights of the subjects were determined independently from two fat prediction equations; namely,

1. Rathbun-Pace (7):

$$\%F = 100\left[\frac{5.548}{\text{sp.gr.}} - 5.044\right]$$

and

2. Keys-Brožek (8):

$$F = \left[\frac{4.201}{D} - 3.813\right]$$

where sp. gr. = specific gravity
D = density

Inherent in the method for measuring total body volume are those errors involved in measurements of gross body volume and residual lung volume as well as unmeasured volume of gas in the gastrointestinal tract. The most unfavorable estimate of error is obtained by adding the maximum estimated range of these three errors. This sum causes an approximate error of about 1 per cent in body density.

Comparison of the correlation coefficients obtained by the use of the data obtained from the two equations is discussed below.

Subjects

Most of the subjects were normal volunteers. Several of the most obese individuals were selected from the hospital files because their chief admission diagnosis was obesity, and because other findings were not considered significant. Most of the procedures for body volume and lung volume determinations were carried out between 11:00 a.m. and noon before lunch or after 4:00 p.m.

RESULTS AND DISCUSSION

Tables I and II give the data for men and women, respectively. Included are the age, height, weight, lean weight, fat weight, circulating red cell volume, calculated plasma volume, calculated blood volume, hematocrit, and body volume. The values for lean and fat weight were obtained using the Rathbun-Pace equation (7). Table III gives the means and standard deviations for the following measures of men and women: Circulating red cell

TABLE III

Blood volumes in normal male and female subjects (Mean ± S.D.)

Measurement	Men (42)*	Women (20)*
Circulating red cell vol. (*Liters*)	2.26 ± 0.37	1.43 ± 0.17
Calculated blood volume (*Liters*)	4.91 ± 0.66	3.49 ± 0.41
Calculated plasma volume (*Liters*)	2.66 ± 0.35	2.06 ± 0.28
Circulating red cell vol. (*ml./kg.*)	28.27 ± 4.11	24.24 ± 2.59
Calculated plasma volume (*ml./kg.*)	33.45 ± 5.18	34.77 ± 3.24
Calculated blood volume (*ml./kg.*)	61.54 ± 8.59	58.95 ± 4.94

* Number of subjects.

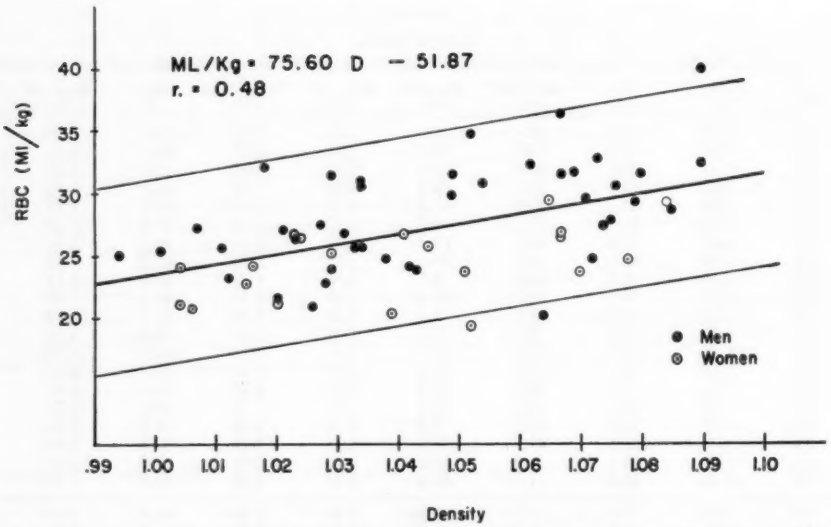

FIG. 1. CIRCULATING RED CELL VOLUME PLOTTED AGAINST BODY DENSITY OF FORTY-TWO MEN AND TWENTY WOMEN

Shown are the regression equation and correlation coefficient, "r." See text for regression equation and correlation coefficients for men and women separately.

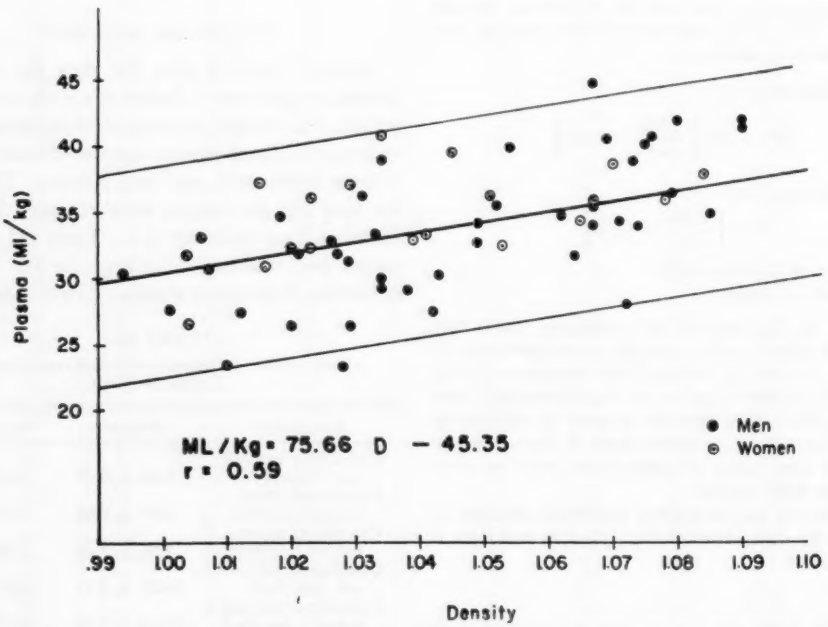

FIG. 2. CALCULATED PLASMA VOLUME PLOTTED AGAINST BODY DENSITY OF FORTY-TWO MEN AND TWENTY WOMEN

Shown are the regression equation and correlation coefficient, "r."

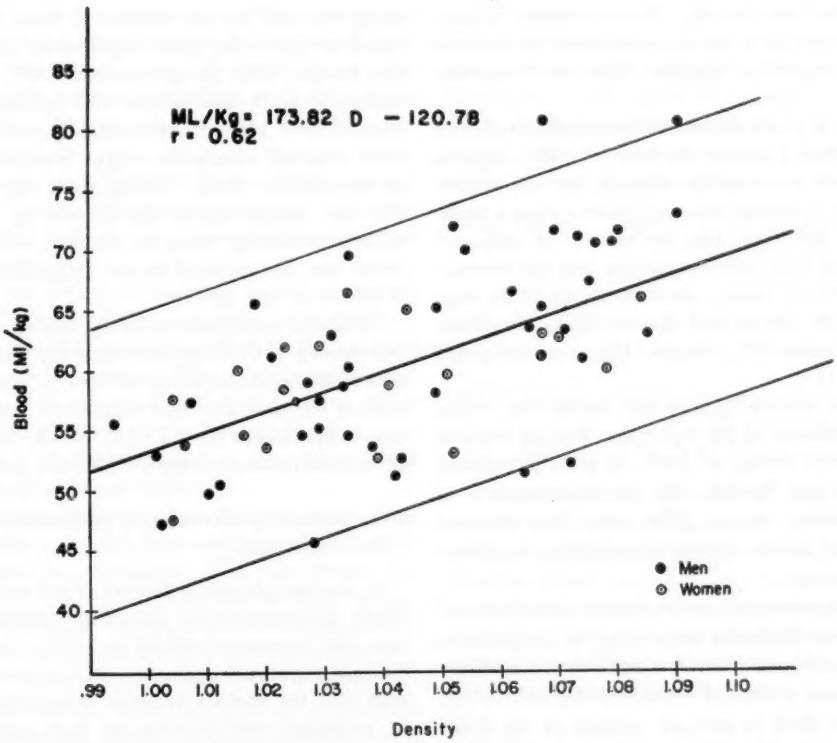

FIG. 3. CALCULATED BLOOD VOLUME PLOTTED AGAINST BODY DENSITY OF FORTY-TWO MEN AND TWENTY WOMEN

Shown are the regression equation and correlation coefficient, "r."

volume, calculated blood volume and calculated plasma volume (liters), as well as the same measures per unit body weight. These values correspond closely to those reported by Berlin, Hyde, Parsons, and Lawrence (9) and Berlin, Hyde, Parsons, Lawrence, and Port (10).

Of particular interest in these data is the extent of the dispersion of the samples about the mean, due, undoubtedly, to the particular population selected for range of obesity. For this group, it appears that diagnosis of an increase in circulating red cell volume requires a value greater than 38 ml. per kg. body weight for men and 29 ml. per kg. body weight for women. Ninety-five per cent of the population lies between 20 and 38 ml. per kg. for men and 19 and 29 ml. per kg. for women. This range is about 29 per cent and 22 per cent of the mean for men and women, respectively.

In Figures 1, 2, and 3 are plotted the various volumes against body density. Also shown is the regression equation for each of these sets of data. The correlation coefficient for red cell volume and body density shown in Figure 1 is 0.48 and is highly significant. However, the distribution of the male and female population above and below the line is apparently not random. For any density there are a greater percentage of the data of women below the regression line for red cells. In fact there are only 3 of 20 of the data of females above the line. When the population is separated into men and women, the following regression equations were found:

Men (42 subjects)
CRCV (ml./kg.) = 80.03 D − 55.51
Women (20 subjects)
CRCV (ml./kg.) = 47.25 D − 24.83

where D = body density. For all values of density, the volume of red cells calculated for women from the regression equation were lower than for men.

Figure 2, which illustrates the calculated plasma volume plotted against the body density, suggests an opposite relationship wherein, for any degree of obesity, a female has more plasma than a male.

These data show that the volume of cells and plasma per unit body weight are less for increasing degrees of obesity, and correspond to the suggestions of Gibson and Evans (11), Gregersen and Nickerson (12), Perera (13), and Hardy and Drabkin (14).

It is of interest to note and discuss the values of extrapolation of the regression lines of Figures 1 and 2 to a density of 0.948—a value considered by Keys and Brožek (8) as representative of human obesity tissue. The value thus obtained for red cell volume of this type of tissue is 20.0 ± 3.7 ml. per kg.

This experimental extrapolated value for red cell volume of obesity tissue may be compared to a value calculated from data such as extracellular fluid content of obesity tissue and the ratio of extracellular fluid to red cell volume in the body. The ratio of extracellular fluid volume to circulating red cell volume calculated from data obtained on 12 healthy adults reported by Hay, Hay, and Jenkins (15) is approximately 6.8. Observations of Keys and Brožek (8) indicate that a mean value of 14.2 per cent of obesity tissue, added when a healthy man gains weight, is accounted for as extracellular fluid. Using, then, the ratio of 6.8, one would expect the circulating red cell volume of obesity tissue to be 20.8 ml. per kg. which may be compared to our extrapolated value of 20.0 ± 3.7 ml. per kg.

Similarly, extrapolation of the line in Figure 2 to a density of 0.948 gives a value for plasma volume of 26.2 ± 4 ml. per kg. of obesity tissue. The ratio of extracellular fluid volume to plasma volume is approximately 5.2 (15) which would give an expected plasma volume of 27.3 ml. per kg.

Data analysis by derivation of multivariant regression equations

It was thought that estimates of red cell volume might be improved by including variables other than the customary weight or surface area in a multiple regression equation. Also it seemed possible that the various available parameters might be evaluated with relation to their appropriate weight or significance. Inspection of the data

TABLE IV

Di- and trivariant correlation coefficients for volumes and lean weight, or fat weight, and lean and fat weight calculated independently from two fat prediction equations

Blood compartment (*liters*)	Population	Lean body mass (*kg.*)		Fat weight (*kg.*)		Lean body mass and fat weight	
		Rathbun-Pace	Keys-Brožek	Rathbun-Pace	Keys-Brožek	Rathbun-Pace	Keys-Brožek
Circulating red cell volume	Total (62)*	.670†	.806	.429	.422		
Calculated blood volume	Total (62)	.719	.840	.392	.388		
Calculated plasma volume	Total (62)	.701	.791	.307	.307		
Circulating red cell volume	Women (20)	.296‡	.559	.518	.522	.759	.759
Calculated blood volume	Women (20)	.476	.733	.497	.518	.890	.891
Calculated plasma volume	Women (20)	.516	.738	.421‡	.448	.862	.856
Circulating red cell volume	Men (42)	.342	.585	.500	.504	.708	.707
Calculated blood volume	Men (42)	.435	.640	.417	.423	.711	.708
Calculated plasma volume	Men (42)	.457	.579	.249‡	.257	.602	.597

* Figures in parentheses represent sample number.
† See Table V for values of r at different levels of significance.
‡ These values for r are not significantly different from zero at 5 per cent level of probability.

shown in Figure 1 suggested that circulating red cell volume of women should be considered separately from that of men. Therefore, the regression models were calculated for men and women separately. Two models of linear regression were investigated for each sex, since there were insufficient data to suggest other than linear relationships. These models were tested for the calculated blood volumes as well as the measured red cell volume.

Both the Rathbun-Pace (7) and Keys-Brožek (8) body fat prediction equations were used for the multivariant analyses which included lean weight and fat weight. The multiple correlation coefficients for these parameters are given in Table IV. Also shown in Table IV are bivariate correlation coefficients for fat or lean weight with blood, plasma or cell volume.

Using the Rathbun-Pace equation more comlex models than just lean weight and fat weight were tested for correlation. The first of these for 42 men included as variables body volume, height, weight, and age. The multiple correlation coefficient for this model was 0.71 and was highly significant with probability $<< 0.01$ of being obtained by chance. However, tests of significance of the individual regression coefficients showed a definitely significant relationship with weight ($P < .05$), a possibly significant relationship with body volume ($P > .10$) and no significant linear relationship with height and age.

The model was then revised to include only weight and body volume and for the 42 men was as follows:

$$\text{Blood Volume (liters)} = 0.123 \text{ weight (kg.)} - 0.091 \text{ body volume (liters)} + 1.987$$
$$\text{with } R = 0.71,\ P << .01,\ s = 0.48$$

Test of individual regression coefficients showed the linear relationship of blood volume to weight even more significant ($P < .02$), while body volume was significant only at the 7 per cent level ($P < 0.07$).

The corresponding equation from data obtained on 42 men for circulating red cell volume was as follows:

$$\text{CRCV (liters)} = 0.044 \text{ weight (kg.)} - 0.026 \text{ body volume (liters)} + 0.72$$
$$\text{with } R = 0.71,\ P < .001,\ s = 0.27$$

The data on 20 women were subjected to the same analyses using the same original model with body volume, height, weight, and age as variables. In contrast to the results obtained from the men's data, the two variables which proved to be significant when tested were age and weight, and the following equation was obtained:

$$\text{Blood Volume (liters)} = 0.039 \text{ weight (kg.)} - 0.0018 \text{ age (yrs.)} + 1.248$$
$$\text{with } R = 0.90,\ P < .01,\ s = 0.19$$

The data from women gave an equation for circulating red cell volume as follows:

$$\text{CRCV (liters)} = 0.0136 \text{ weight (kg.)} - 0.002 \text{ age (yrs.)} + 0.707$$
$$\text{with } R = 0.78,\ P < .001,\ s = 0.12$$

A second model included as variables, lean weight, fat weight and surface area. Test of regression coefficients for surface area of the multiple equation showed no significant linear relationship to blood volume and circulating red cell volume for data of both men and women and was consequently deleted from the model. The four resulting equations were as follows:

Men

1) Blood Volume (liters) = .042 lean wt. (kg.) + 0.025 fat wt. (kg.) + 1.92

2) CRCV (liters) = 0.021 lean wt. (kg.) + 0.016 fat wt. (kg.) + 0.70

Women

1) Blood Volume (liters) = 0.045 lean wt. (kg.) + 0.034 fat wt. (kg.) + 1.02

2) CRCV (liters) = 0.014 lean wt. (kg.) + 0.013 fat wt. (kg.) + 0.63

Tests of the regression coefficients showed highly significant linear relationship between these volumes and lean weight and fat weight, having P values $<< .001$ in every case except one: circulating red cell volume (liters) and lean weight (kg.) in women had $P > .01$.

Thus, these considerations suggest that there is a high degree of correlation between lean weight and red cell volume as well as plasma volume and blood volume. Moreover, it is equally strongly suggested that there is a high degree of correlation

of these values with fat weight. It is interesting that the P value for the significance of the coefficient for fat weight in women ($< .001$) was better, than that for lean, $< .01$. The tendency in the literature and in discussions has been to emphasize the quantity of blood allocated to lean tissue. It is true that in men the quantity of blood apparently associated with lean tissue is about twice that of fat; but in women this difference of fat and lean is much less.

The analyses do indicate very strongly that the correlation coefficient obtained by trivariant analysis when lean and fat weight are considered together as variables have higher values than when only lean weight is considered. Table IV shows a higher correlation coefficient for every case when fat is included as a variable. In every case, there was also a slight decrease in dispersion. Thus, fat weight as well as lean weight must be considered when predicting blood volumes since lean weight alone gives a poorer correlation.

However, the multiple correlation coefficients of the trivariate analyses of fat weight, and lean weight with each of the volumes is no better than the bivariate correlation coefficients of total weight, or surface area, or logarithm of the body weight with each of the volumes. These last values are shown in Table V. This raises the possibility that these parameters are basically related to blood volume but in themselves have little to do with the normal variations among individuals. An important question in this relation is, "What is the validity and reliability with which red cell volume may be measured with Cr^{51}?" Evaluation of the error of this method is as difficult as it is important. It would be relatively simple to evaluate the error of the method for an *in vitro* system; however, the error associated with mixing is not apparently soluble at this time. Blood samples for measurement of the chromium-51 concentration were drawn at five and ten minutes. Inspection and comparison of the counting rates of the two samples did not suggest any significant systematic difference. Usually the difference in the values was less than the error of the analysis. The count rate of the second sample from markedly obese individuals was not less than that of their first sample. Therefore differences in mixing in obese individuals are likely to be evident in a comparison of samples drawn before five minutes. From these considerations, it is unlikely that the circulating red cell volume of obese individuals was incorrectly estimated because of prolonged mixing. It is our opinion that the apparent variation of 25 per cent between individuals is not the result of error of the method, but involves other parameters. Berlin, Hyde, Parsons, and Lawrence (9) found that with repeat determinations in a series of 24 P^{32} tagged cell volumes, the maximum variation was 7.4 per cent and the average variation was 5.7 per cent.

For any density, the amount of circulating red cell mass per kilogram of tissue is less in women than in men. Therefore, the difference in circulating red cell mass in men and women is on a ba-

TABLE V

Comparison of correlation coefficients for blood volumes and surface area, total body weight, and log of body weight

Blood compartment (*liters*)	Population	Sample number	Surface area M^2	Total body weight *kg.*	Log of body weight *log kg.*
Circulating red cell volume	Total	62	.876*	.637	.861
Calculated blood volume			.867	.891	.890
Calculated plasma volume			.824	.734	
Circulating red cell volume	Women	20	.753	.763	
Calculated blood volume			.871	.873	
Calculated plasma volume			.832	.825	
Circulating red cell volume	Men	42	.604	.697	
Calculated blood volume			.761	.673	.690
Calculated plasma volume			.578	.520	

* For 62 subjects r is significantly different from zero at the 5 per cent level of probability if $r = 0.25$ and at the 1 per cent level if $r = 0.32$. For 42 subjects similar values of r are 0.30 and 0.39, respectively. For 20 subjects similar values of r are 0.42 and 0.54, respectively. All r's in the table are significantly different from zero at 1 per cent level of probability.

sis other than obesity alone. The role of gonads in erythropoiesis has been reviewed by Van Dyke, Contopoulos, Williams, Simpson, Lawrence, and Evans (16), and additional data were presented showing that testosterone causes increased cell production and a greater circulating red cell volume while estrogen works an opposite effect (16). Hollingsworth and Hollingsworth (17) recently have collected data on Cr^{51} tagged red cell survival in women which suggest that cell longevity is less in women than in men.

The value obtained by extrapolation for the amount of blood in obesity tissue if applied to the lean individual who becomes very obese gives impressive increases in blood volume. For example, if a lean 60 kilogram man gains 40 kilograms of obesity tissue, it is implied that his former blood volume of about 4000 milliliters would be expected to increase to about 5200 milliliters. Thus, 1200 milliliters of blood would be associated with his obesity tissue.

The studies of Jones (18) indicate that fat tissue is relatively poorly perfused with blood. Taylor, Brožek, and Keys (19) have shown that the cardiac output does not increase with increases of obesity tissue. Contrariwise they showed a trivariate regression equation relating cardiac output to lean body mass and fat, and found that the coefficient for fat is negative. The same authors showed that the arterial-mixed venous oxygen difference was positively correlated with obesity.

The data here indicate that the amount of blood per unit obesity tissue is relatively large. Along with the findings of others regarding perfusion rates and cardiac output, these data emphasize the possible magnitude of relatively slow moving pools of blood in the obese individual and are pertinent to speculation concerning the increased incidence of death due to thromboembolism in individuals having these characteristics.

SUMMARY

Circulating red cell volume and body density were measured in a group of men and women. Multiple regression equations were derived showing that circulating red cell volume is significantly correlated with fat as well as lean tissue. The amount of blood associated with obesity tissue is apparently about two-thirds that of lean tissue. Since blood perfusion of obesity tissue is relatively low, this implies that obesity results in the presence of relatively large quantities of blood having low turnover rates. There was little increase in ability to estimate red cell volume on the basis of such equations when applied to an individual of this sample over the estimate based on weight alone. Women were found to have a circulating red cell volume per unit weight less than that of men for any degree of obesity.

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THE EFFECT OF CERTAIN CHELATING SUBSTANCES (EDTA) UPON CHOLESTEROL METABOLISM IN THE RAT[1]

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There is increasing use in biology and medicine of certain salts of ethylenediamine tetraacetic acid (EDTA), substances which can form stable complexes with multivalent positive ions. Certain such chelating agents have been reported (1–3) to affect cholesterol metabolism. It therefore was believed of interest to study the nature of these possible effects.

I. *The Effect of Ingested Salts of EDTA on Plasma Cholesterol of Rats Fed Excess Cholesterol and Cholic Acid*

Methods

A series of 23 rats (I) was divided into two groups. Stock laboratory diet was fed to 15 rats while 8 rats were fed the same ration with added disodium EDTA[2] (4 per cent of diet).

Another series of 32 rats (II) was divided into four groups which were fed stock laboratory ration to which was added powdered cholesterol (2 per cent of diet) and cholic acid (1 per cent of diet), previously shown to induce chronic hypercholesteremia in rats (4, 5). Eight control rats ingested only this ration. The disodium salt of EDTA (4 per cent of diet) was added to the ration of 8 rats, tetrasodium EDTA (4 per cent of diet) to that of 8 other rats, and disodium calcium EDTA (4 per cent of diet) to the ration of the remaining 8 rats.

A third series of 30 rats (III) was fed stock ration with a larger amount of added powdered cholesterol (4 per cent of diet) and cholic acid (1 per cent of diet). The rats were divided into four groups. The basic ration was fed to 6 control rats while 8 rats in each of the three other groups ingested the same ration containing added salts of EDTA as in the preceding series of rats.

The daily food consumption of all rats was measured for 7 days. The rats of the first two series were bled after 14 days and those of the third series after 21 days. In each instance the plasma cholesterol was determined on an alcohol-acetone extract according to the method of Zlatkis, Zak, and Boyle (6).

RESULTS

In Table I it can be seen that most rats ingesting diets with EDTA supplements exhibited moderate loss of weight. This could not be ascribed to diminished food intake since these rats each ingested from 16 to 21 grams of food per day, an amount essentially the same as that ingested by the control animals.

The ingestion of ration supplemented with cholesterol and cholic acid, as previously noted (4, 5), induced progressive chronic hypercholesteremia in the rats of Series II and III. As shown in Table I the magnitude of this hypercholesteremia was increased by the addition to the diet of each of the salts of EDTA, but was greatest in the animals fed disodium EDTA. In contrast, the rats ingesting ration containing disodium EDTA without added cholesterol or cholic acid (Series I) failed to exhibit any rise of plasma cholesterol.

II. *The Effect of Injected Disodium EDTA on Plasma Cholesterol of Rats Fed Excess Cholesterol*

The following experiment was performed in order to determine if the above augmenting effect of *ingested* EDTA on the plasma cholesterol of rats fed excess cholesterol also occurred when EDTA was given parenterally and when cholic acid was omitted from the ration.

Methods

A series of 24 rats was divided into three groups. Stock ration containing added powdered cholesterol (4 per cent of diet) was fed to all rats, but cholic acid was not added. One group of 8 rats (I) served as controls. Disodium EDTA (4 per cent of diet) was added to the ration of another group of 8 animals (III). The remaining 8 rats (II) ingested the cholesterol-supplemented ration and each rat was injected subcutaneously with a solution of 30 mg. of disodium EDTA daily (pH 7.25). For control purposes a final group of 8 rats (IV) was fed stock ration. After 14 days all rats were bled for determination of plasma cholesterol.

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[2] We wish to express our appreciation to Dr. Joseph A. Morrell of the R. J. Strasenburgh Company for supplying the EDTA salts used in this investigation.

TABLE I

Effect of ingested salts of EDTA on plasma cholesterol of rats fed excess cholesterol and cholic acid

Series	Dietary supplement	No. of rats	Average weight		Average plasma total cholesterol	
			Initial (gm.)	Final (gm.)	Day 14 (mg./100 ml.)	Day 21 (mg./100 ml.)
I. *Rats fed stock diet without added cholesterol or cholate*						
	Control	15	150	172	62 (48–73)*	—
	Disodium EDTA (4%)	8	155	134	57 (29–65)	—
II. *Rats fed stock diet containing 2% cholesterol, 1% cholate*						
	Control	8	142	177	171 (71–305)	—
	Disodium EDTA (4%)	8	146	122	719 (486–880)	—
	Tetrasodium EDTA (4%)	8	153	124	286 (172–376)	—
	Disod. calcium EDTA (4%)	8	158	178	211 (125–376)	—
III. *Rats fed stock diet containing 4% cholesterol, 1% cholate*						
	Control	6	150	166	—	319 (237–420)
	Disodium EDTA (4%)	8	148	121	—	861 (377–1455)
	Tetrasodium EDTA (4%)	8	154	131	—	669 (343–1165)
	Disod. calcium EDTA (4%)	8	172	152	—	598 (383–1160)

* Range of values.

Results

In Table II it can be seen that the addition of excess cholesterol alone to the diet (Group I) induced a slight rise of plasma cholesterol (average: 93 mg. per 100 ml.). A rise of plasma cholesterol of similar magnitude (average: 88 mg. per 100 ml.) occurred in the rats (Group II) fed the same ration and injected daily with disodium EDTA. In contrast, a significantly greater rise of plasma cholesterol (average: 190 mg. per 100 ml.) was induced by addition of disodium EDTA to the cholesterol-supplemented ration (Group II).

III. *The Effect of Disodium EDTA on Intestinal Absorption of Cholesterol*

The previous demonstration (7) that exogenous cholesterol, after its intestinal absorption, is transported into the systemic circulation by the thoracic duct lymph provides a means of quantitating the

TABLE II

Effect of injected disodium EDTA on plasma cholesterol of rats fed excess cholesterol

Group	Disodium EDTA supplement	No. of rats	Average weight		Average plasma total cholesterol* (mg./100 ml.)
			Initial (gm.)	Final (gm.)	
Rats fed stock ration with added cholesterol (4%)					
I	None	8	230	236	93 (81–107)†
II	Injection of disodium EDTA (30 mg. subcutaneously daily)	8	229	226	88 (81–109)
III	Disodium EDTA added to diet (4%)	8	215	204	190 (103–382)
Rats fed stock ration					
IV	None	8	250	270	60 (44–79)

* Day 14.
† Range of values.

effect of the chelating agent upon intestinal absorption of cholesterol.

Methods

As previously described (7) thoracic duct cannulation was performed in a series of rats following an overnight fast. The lymph was collected for 24 hours following the stomach feeding of 50 mg. of cholesterol dissolved in 1.5 ml. of olive oil. Twelve rats served as controls while 17 other rats received a similar stomach feeding but also containing 50 mg. of disodium EDTA. In each instance the cholesterol content of the lymph was then determined in the 24-hour sample of lymph.

Results

In Table III it can be seen that the fraction of absorbed cholesterol was increased by feeding disodium EDTA with the test dose of cholesterol. Thus the control rats absorbed an average of 18.0 mg. of cholesterol in 24 hours while the rats also fed disodium EDTA absorbed an average of 26.0 mg. It is important to point out that an even greater difference may have been obtained if the EDTA would have dissolved in the oily solution of cholesterol.

IV. *The Possible Effect of Disodium EDTA on Disappearance of Cholesterol from the Plasma*

The previous studies indicated that the addition of salts of EDTA to the diet induced hypercholesteremia by augmenting the intestinal absorption of cholesterol. The following experiment was done to determine if the chelating agent also acted by impeding the usual disappearance of cholesterol from the plasma.

A. *The possible effect of disodium EDTA on the disappearance of cholesterol from the plasma of dietary-induced hypercholesteremic rats*

Methods

A series of 25 rats was fed stock ration containing added powdered cholesterol (2 per cent) and cholate (1 per cent). After 10 days all rats were bled for determination of plasma cholesterol and then were placed upon a fat-and-sterol-free diet. The rats were divided into 3 groups of which one group (I) of 6 rats served as controls, and a second group (II) of 11 rats was injected subcutaneously with 50 mg. of disodium EDTA daily. Disodium EDTA was added to the ration (5 per cent of diet) of the third group (III) of 8 rats. All rats were bled for determination of plasma cholesterol 7 days later.

Results

Table IV shows that hypercholesteremia again was induced by feeding excess cholesterol and cholate (4, 5). When the fat-and-sterol-free diet subsequently was fed for 7 days, the magnitude of the previously induced dietary hypercholesteremia progressively diminished. The rate of disappearance of the excess cholesterol from the plasma was not found to be diminished by the dietary or parenteral administration of disodium EDTA.

B. *The possible effect of disodium EDTA on the disappearance of injected "physiological" cholesterol from the plasma*

Methods

Hypercholesteremic rat plasma was prepared by ligating the bile ducts of a series of rats. For 3 days, each rat was starved except for a daily stomach feeding of 100 mg. of cholic acid. As previously found (8) such rats develop marked hypercholesteremia of endogenous

TABLE III

Effect of disodium EDTA on intestinal absorption of cholesterol in rats

Disodium EDTA supplement	No. of rats	Average weight (gm.)	Lymph (0 to 24 hours)*		
			Average volume (ml.)	Average cholesterol	
				Concentration (mg./100 ml.)	Content (mg./24 hr.)
None	12	249	21.9 (15.0–33.0)†	84.0 (41.0–109.0)	18.0 ± 1.77‡ (12.5–30.0)
50 mg.	17	278	28.0 (19.0–40.0)	94.0 (53.0–218.0)	26.0 ± 1.9 (13.0–48.0)

* Following stomach feeding of 50 mg. cholesterol dissolved in 1.5 ml. olive oil.
† Range of values.
‡ Standard error of the mean.

TABLE IV

Effect of disodium EDTA on regression of previously induced dietary hypercholesteremia in rats*

Group	Dietary regimen	Disodium EDTA injections	No. of rats	Average weight		Average plasma total cholesterol		Average decrease of plasma cholesterol (*mg./100 ml.*)
				Initial (*gm.*)	Final (*gm.*)	Day 0 (*mg./100 ml.*)	Day 7 (*mg./100 ml.*)	
I	Fat and sterol-free	None	6	206	228	143 (109–201)†	94 (74–111)	49 ± 13.9‡
II	Fat and sterol-free	50 mg. subcutaneously daily	11	234	234	166 (100–285)	112 (74–1637)	54 ± 10.8
III	Fat and sterol-free: disodium EDTA (5%)	None	8	241	200	186 (132–342)	110 (62–177)	76 ± 15.7

* By feeding diet containing 2 per cent cholesterol and 1 per cent cholate for 10 days.
† Range of values.
‡ Standard error of the mean.

origin. The rats were bled, their plasma pooled, and the pooled plasma was found to contain 15 mg. of cholesterol per 3-ml. aliquot.

A series of 5 rats was subjected to cannulation of their lumbar veins with polyethylene tubes and then placed in restraining cages. Each rat was injected intravenously with 5 mg. of disodium EDTA and 3 ml. of the hypercholesteremic rat plasma containing 15 mg. of excess "physiological" cholesterol. Then, by means of a special apparatus, each rat received 6 ml. of a solution (pH 7.25) containing 50 mg. of disodium EDTA by constant intravenous infusion over an 18-hour period. All rats were bled for plasma cholesterol immediately after injection of the excess cholesterol load and again in 18 hours. Another series of 5 control rats was treated similarly except that 5 per cent glucose was used as the intravenous infusion fluid.

Results

Immediately after injection of the excess cholesterol load, the experimental rats had an average plasma cholesterol of 201 mg. per 100 ml. (Range: 158 to 240) and the control rats averaged 192 mg. per 100 ml. (Range: 140 to 232). After 18 hours the average plasma cholesterol of the two groups were, respectively, 77 mg. per 100 ml. (Range: 72 to 82) and 76 mg. per 100 ml. (Range: 60 to 90). Thus the intravenous infusion of disodium EDTA was not found to impede the usual disappearance of the excess cholesterol from the plasma.

C. *The possible blocking effect of disodium EDTA on the egress of endogenously synthesized cholesterol from the plasma*

Methods

Previously it was shown that cholate (8, 9), Triton WR 1339 (10), hypothyroidism (11), and the induction of nephrosis (12) in the rat are each capable of impeding the usual transfer of endogenously synthesized cholesterol from the plasma into the liver. In order to determine whether disodium EDTA also was capable of such "blocking" effects it was injected intravenously into hyperthyroid as well as control rats. The entrance of newly formed cholesterol into the plasma is markedly accelerated in the hyperthyroid state due to a considerably increased rate of hepatic synthesis of cholesterol (11). Thus the ability of any substance even to retard slightly the usual egress of newly synthesized cholesterol from the plasma would, of course, be more strikingly shown by administering it to animals with an accelerated turnover of plasma cholesterol such as occurs in hyperthyroid rats. A series of 6 rats was made severely hyperthyroid by adding powdered thyroid substance to their diet (0.3 per cent) for 3 weeks (11). A second series of 8 rats remained euthyroid. Disodium EDTA (30 mg.) was injected subcutaneously daily for 3 days into all rats and then 50 mg. of the chelating substance, contained in 7 ml. of solution, was given to each rat by continuous intravenous infusion over the ensuing 24-hour interval into a cannulated lumbar vein. At the end of this 24-hour interval all rats were bled for determination of plasma cholesterol.

Results

The plasma cholesterol of the 8 euthyroid rats averaged 62 mg. per 100 ml. (Range: 48 to 73) and that of the 6 hyperthyroid animals averaged 59 mg. per 100 ml. (Range: 45 to 72). The failure of the plasma cholesterol to rise following the 24-hour intravenous injection of disodium EDTA in either group of rats indicates that the chelating agent does not prevent the usual egress of cholesterol from the plasma, even when the rate of synthesis of the plasma cholesterol is considerably increased (11).

V. *The Possible Effect of Disodium EDTA upon the Hepatic Deposition of Cholesterol*

In order to determine if the chelating agent is capable of inducing hypercholesteremia by blocking the deposition of absorbed cholesterol into the liver, the following experiment was performed.

Methods

A series of 14 rats was fed stock ration containing added cholesterol (4 per cent) and cholate (1 per cent). Disodium EDTA (4 per cent) was added to the ration of 8 rats and 6 other rats served as controls. The animals were sacrificed after 21 days, at which time the concentrations of cholesterol in the plasma and liver were determined.

Results

In Table V it can be seen that the hypercholesteremia induced by feeding excess cholesterol and cholate again was considerably augmented by addition of disodium EDTA to the diet and again occurred in spite of the loss of weight induced by the chelating agent. The cholesterol concentrations of the livers of both groups of rats were markedly increased, the highest concentrations being observed in the rats whose ration contained the disodium EDTA. These rats exhibited a lower total liver cholesterol content due to their diminished liver weight which was associated with the loss of total body weight induced by the EDTA. It seems clear that the chelating agent did not impede the egress of cholesterol into the liver from the plasma.

VI. *The Effect of Disodium EDTA upon the Rate of Hepatic Synthesis of Cholesterol*

The next experiments were performed in order to determine whether administration of the chelating agent was capable of significantly altering the rate of cholesterol synthesis by the liver. Two different techniques were used to study this problem.

A. *Effect of disodium EDTA upon bile cholesterol content*

Methods

As previously shown (13) the rate of excretion of biliary cholesterol provides an index of the rate of hepatic synthesis of cholesterol in the rat. The reasons underlying the validity of this technique have been fully discussed elsewhere (14, 15). This index fails to follow the rate of incorporation of C^{14} acetate with cholesterol in all cases. The discrepancy appears particularly great when cholesterol is fed (16). Fredrickson, Loud, Hinkelman, Schneider, and Frantz (17) also believed that a discrepancy existed between the rate of hepatic synthesis as suggested by the biliary index and that observed by their tracer measurements in the bile ligated rat. These authors so believed because they found the rate of hepatic synthesis of cholesterol to be increased as measured by tracer technics whereas the biliary index showed no such increase (8). However, our own later tracer measurements in this same condition (9) as well as those of Landon and Greenberg (18) agree with the biliary index in that they also found no increase in the rate of hepatic synthesis of cholesterol in bile ligated rats. These latter authors also found unequivocal evidence that the cholesterol present in bile was always newly synthesized as measured by tracer technics. Furthermore, after the period of active incorporation of C^{14} acetate into cholesterol had ceased, the cholesterol in bile was not radioactive although such labelled cholesterol was in abundance both in the blood and other tissues of the animal. All of this evidence, of course, suggests that the cholesterol found in bile is a portion or increment of cholesterol, newly synthesized in the liver. This in turn supports its usefulness as an index of the hepatic rate of cholesterol synthesis.

The bile ducts of 17 rats were cannulated and the 24-hour excretion of bile collected and analyzed for its

TABLE V

Effect of disodium EDTA on hepatic cholesterol content of rats fed hypercholesteremic diet for 21 days*

Dietary regimen	No. of rats	Average weight		Average plasma total cholesterol (*mg./100 ml.*)	Liver		
		Initial (*gm.*)	Final (*gm.*)		Average dry weight (*gm.*)	Average cholesterol	
						Concentration (*mg./100 gm.*)	Content (*mg.*)
Control*	6	150	166	319 (237–420)†	3.29 (3.12–3.58)	14,850 (14,050–16,400)	488 (449–522)
Control* disodium EDTA (4%)	8	148	121	861 (377–1455)	2.20 (1.64–2.83)	18,070 (16,350–19,800)	396 (287–463)

* Stock ration containing 4 per cent cholesterol, 1 per cent cholate.
† Range of values.

cholesterol content as previously described (13). Nine rats received a daily subcutaneous injection of 30 mg. of disodium EDTA for 3 days prior to collection of bile and during the 24-hour period of collection of bile each rat also received 7.2 ml. of solution (pH 7.25) containing 50 mg. of disodium EDTA by continuous intravenous injection into a cannulated lumbar vein. The remaining 8 rats served as controls and during the bile collection interval each rat received a continuous intravenous injection of 5 per cent glucose solution. Plasma cholesterol was determined in all rats at the end of the collection period.

Results

As shown in Table VI, the subcutaneous and intravenous administration of disodium EDTA did not alter the plasma cholesterol levels. The concentrations of biliary cholesterol and the 24-hour biliary excretion of cholesterol also were not altered significantly by the administration of the chelating agent. Indeed the average daily excretion of cholesterol was of slightly lower magnitude in the rats treated with the chelating agent.

B. *The effect of disodium EDTA on the rise of plasma cholesterol in rats injected with Triton WR-1339*

The possible effect of the chelating agent on cholesterol synthesis by the liver also was studied by the following technique. Previously it was shown that the endogenous source of the plasma cholesterol is the liver (15) and that injection of a sufficient amount of Triton WR-1339 into the rat seriously impedes for at least 24 hours the usual egress from the plasma of any newly formed cholesterol following its discharge into the blood stream from the liver (10). Thus, the unique property of Triton to impede the normal rate of egress of cholesterol from the plasma offers a means of estimating the amount of cholesterol discharged into the blood by the liver. This latter conclusion has been questioned by Frantz and Hinkelman (19) who found the rate of incorporation of tracer doses of C^{14} labelled acetate into cholesterol to be increased after administration of Triton. However, we note findings by Landon and Greenberg (18) opposed to those of Frantz and Hinkelman (19) concerning the rate of incorporation of C^{14} acetate into cholesterol in another condition, that of bile duct ligation in the rat. Furthermore, Frantz and Hinkelman (19) have cited findings obtained by others as well as themselves concerning the normal rate of synthesis of cholesterol by the rat which show this rate to be too low to account for the cholesterol rise in the blood after Triton administration. Other workers such as Hutchens, Van Bruggen, and West (20) and Landon and Greenberg (18) may be cited who found by similar technics more rapid normal rates of "synthesis" which are adequate to account for the blood cholesterol rise after Triton without the necessity of postulating large increases in synthesis rate. It also should be pointed out that there is agreement between the findings obtained in our laboratory (10) and that of Frantz and Hinkelman (19) concerning the quantitative amounts of cholesterol in plasma and liver before and after administration of Triton to rats. Both groups agree that no change occurs in the *total* amount of cholesterol present in the liver (or other tissues) while the amount of cholesterol present in the blood does increase substantially following Triton injection. The rise in blood cholesterol therefore affords a means for estimating the minimal net amount of cholesterol

TABLE VI

Effect of disodium EDTA upon biliary cholesterol excretion

Type of rat	No. of rats	Average weight (gm.)	Average plasma total cholesterol (mg./100 ml.)	Bile (0 to 24 hours)		
				Average volume (ml.)	Average cholesterol	
					Concentration (mg./100 ml.)	Content (mg./24 hours)
Control	8	230	62.0 (48–73)	13.9 (10.8–18.1)†	22.5 (10.5–30.0)	3.2 (1.4–5.4)
Treated with disodium EDTA*	9	216	50.3 (49.0–59.0)	13.0 (10.8–16.3)	23.3 (11.0–39.0)	2.7 (1.5–5.4)

* See text.
† Range of values.

which was discharged into the blood during the period in which the rise occurred, and this is so regardless of the possibilities for free exchange of cholesterol molecules between plasma and liver shown by tracer studies. In other words, the finding of free exchange between liver and plasma cholesterol following Triton injection fails to explain the quantitative accumulation of cholesterol in the plasma which it induces and does not negate the conclusion that Triton blocks the normal metabolic removal of cholesterol. The actual amount of cholesterol discharged into the blood during the study period will be somewhat higher than indicated by the net rise in blood cholesterol because some is destroyed and excreted during the period of observation (10).

Methods

Five of 10 rats (Av. wt.: 177 gm.) were injected subcutaneously with 30 mg. of disodium EDTA daily for 3 days and then each received through a lumbar vein cannula a continuous intravenous injection of 7 ml. of solution containing 60 mg. of the chelating agent (pH 7.25) during the ensuing 24-hour interval. At the same time 5 rats (Av. wt.: 181 gm.) served as controls, each receiving an injection of 7 ml. of 5 per cent glucose solution. Subsequent to cannulation of the lumbar vein each rat was injected intravenously with 100 mg. of Triton WR-1339 in a 10 per cent solution. All rats were bled for determination of plasma cholesterol 24 hours later.

Results

The injection of Triton led to marked hypercholesteremia in the control rats, with an average plasma cholesterol concentration of 354 mg. per 100 ml. (Range: 285 to 428) (S.E. Mean: ± 32.5) after 24 hours. The rats which also received disodium EDTA exhibited an average plasma cholesterol of 393 mg. per 100 ml. (Range: 319 to 488) (S.E. Mean: ± 34.6). The difference between the average levels in the two groups does not appear to be significant.

DISCUSSION

The preceding observations indicate that salts of EDTA are capable of augmenting to a considerable degree the otherwise slight hypercholesteremia which occurs in rats fed excess cholesterol. In the first experiment (Table I) it was found that the disodium salt of EDTA exerts a much more striking effect in this regard than does the tetrasodium or disodium calcium salt of EDTA.

Previously it was shown that progressive chronic hypercholesteremia occurs in rats when cholesterol and cholic acid are added to the diet (4, 5), due to an increase in the fraction of absorbed cholesterol induced by cholate (9). Addition of disodium EDTA to a diet containing excess cholesterol (Table II) augmented the magnitude of the hypercholesteremia in a fashion similar to that of cholate. Much greater hypercholesteremia occurred in cholesterol-fed rats when cholate and the chelating agent were both added to the ration (Table I). On the other hand, hypercholesteremia was not induced by feeding disodium EDTA in the absence of excess dietary cholesterol (Table I) and the augmenting effect of ingested disodium EDTA upon the hypercholesteremia occurring in the cholesterol-fed rats was not similarly observed (Table II) when the chelating substance was given parenterally. This indicated that cholesterol absorption is increased by salts of EDTA, a conclusion which was substantiated by determining the effect of disodium EDTA upon the intestinal absorption of cholesterol (Table III).

These studies indicate that salts of EDTA markedly increase the hypercholesteremia which occurs in rats fed excess cholesterol, a propensity similar to that of cholic acid (5, 9). These chelating substances, like cholic acid, have surface active effects and are capable of increasing the detergency of surfactants. It seems possible that the effect of both substances upon cholesterol absorption may be related at least in part to their surface active properties. The difference in magnitude of the effects of the various salts of EDTA is probably due to differences in their pH, solubilities, and other properties.

It was of interest to determine if other phases of cholesterol metabolism also were altered by the EDTA salts in such a manner as to contribute to its hypercholesteremic effects. The effect of disodium EDTA upon the rate of hepatic synthesis of cholesterol, the primary source of the plasma cholesterol (15), was studied, the data indicating that the chelating agent did not alter significantly the rate of cholesterol synthesis by the liver. These results are contrary to the conclusion reached by Curran (3) who reported that the incorporation of labelled acetate into cholesterol

by small amounts of surviving liver obtained from two animals was increased by EDTA compared to his control value.

The effect of disodium EDTA upon the egress of cholesterol from the plasma also was studied. No evidence was found that the chelating agents block the usual transfer of cholesterol from the plasma. Thus it was shown that in the absence of excess dietary cholesterol the plasma cholesterol was not elevated either by oral (Table I) or subcutaneous (Table II) administration of disodium EDTA or by its intravenous infusion (Table VI), even when the rate of turnover of the plasma cholesterol was markedly increased by the presence of an hyperthyroid state (11). Furthermore, when hypercholesteremia was induced by dietary means, and subsequently allowed to regress by placing the animals on a fat-and-sterol-free diet, the rate of disappearance of the excess cholesterol from the plasma was not diminished either by oral or subcutaneous administration of disodium EDTA (Table IV). Moreover, it also was found that the chelating agent did not impede the usual disappearance from the plasma of an injected load of excess "physiological" cholesterol of endogenous origin. Finally, it was found that the disodium EDTA did not block the entrance of cholesterol from the plasma into the liver (Table V), also indicating that the hypercholesteremic effect of the chelating agent could not be ascribed to any "blocking" effect upon the usual egress of cholesterol from plasma into the liver. These results are not in agreement with the conclusions reached by Uhl, Brown, Zlatkis, Zak, Myers, and Boyle (2) who found that the subcutaneous administration of sodium EDTA augmented dietary-induced hypercholesteremia in rabbits in a fashion similar to that induced by its oral administration. However, the intake of food by their rabbits was not reported and the relatively small plasma cholesterol differences in their groups of rabbits were not subjected to statistical evaluation. These authors also observed that both oral and parenteral sodium EDTA protected against deposition of dietary-derived cholesterol in the rabbit's liver, an effect which we could not confirm in the rat. Our observations therefore do not substantiate their conclusion that the effect of the chelating agent upon the plasma cholesterol is mediated by interference with the transfer of cholesterol from plasma into the liver.

CONCLUSION

The effects of various salts of EDTA upon cholesterol metabolism were studied in rats. It was found that the oral administration of these substances markedly increases dietary-induced hypercholesteremia by augmenting the intestinal absorption of cholesterol. The chelating agents were not found to alter the hepatic synthesis of cholesterol in rats or to interfere with the usual egress of cholesterol from the plasma or to block the deposition of cholesterol in the rat's liver.

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OBSERVATIONS ON THE METABOLIC EFFECTS OF THE CARBONIC ANHYDRASE INHIBITOR DIAMOX®: MODE AND RATE OF RECOVERY FROM THE DRUG'S ACTION

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Ingestion of certain unsubstituted sulphonamides causes an increased excretion of sodium, potassium, and bicarbonate ions. The effects are probably due to reduction of the quantity of hydrogen ions available for secretion by the renal tubules, with consequent impairment of the $Na^+ - H^+$ exchange postulated by Pitts and Alexander as being responsible for the tubular reabsorption of bicarbonate (1).

Schwartz (2) suggested that the natriuretic effect of carbonic anhydrase inhibitors might prove to be of therapeutic value in patients with sodium retention and congestive heart failure, and for this purpose Miller, Dessert, and Roblin tested the inhibitory potency of a number of heterocyclic sulphonamides *in vitro* (3). Among the most powerful of their compounds was Diamox®, 2-acetylamino-1, 3, 4-thiadiazole-5-sulphonamide, the substance used in the present investigation.

The effects of Diamox® on the urinary excretion of water and electrolytes have been described by Nadell (4), and by Friedberg, Taymor, Minor, and Halpern (5) who noted that the drug's action is transient. The present study was undertaken primarily to determine the mechanism and rate of recovery from the disturbance of acid-base balance produced by the drug.

MATERIAL AND METHODS

Two normal adults, referred to as Subjects 'A' (male, 34 years, wt. 72 kg.) and 'B' (female, 31 years, wt. 61 kg.) maintained a fixed intake of food and fluid for 13 and 16 days, respectively. After control periods of six and seven days, Diamox® was ingested for four and three days in a dosage of 0.25 g. 6-hourly. Measurements were continued for recovery periods of three and six days, respectively. All urine was collected under oil in two 12-hour periods for each day, and a complete analysis was made of its acid-base composition. In addition more limited urinary studies were carried out on four normal adults who ingested Diamox® at daily intervals for 3 to 5 days. Blood samples were taken at approximately 10.00, 16.00, and 22.00 hours each day and all urine collected under oil over corresponding intervals. Observations were also made on eight patients with heart failure (six cor pulmonale, two mitral stenosis), seven of whom ingested 0.25 g. of Diamox® 6-hourly, the eighth 0.2 g. 4-hourly. Urine was collected over 4 to 6-hour periods until bicarbonate excretion ceased.

Blood samples were taken from an arm vein in which the blood had been rendered arterial in character by immersion of the limb in water at 118°F for ten minutes. Plasma for carbon dioxide analysis was obtained by anerobic centrifugation of the blood in the syringes employed for collection. Analytic methods used were as follows:

Sodium, Potassium	Flame Photometer
Ammonia	Aeration and titration (6)
Titratable acid	Electrometric titration to pH. 7.4
Magnesium	Titan yellow colorimetric (7)
Chloride (urine and plasma)	Electrometric titration (8)
Calcium	Oxalate precipitation, permanganate titration (9)
Phosphate	Molybdate colorimetric (10)
Sulphate	Benzidine titrimetric (11)
Organic acids	Titration (12)
Total carbon dioxide (urine and plasma)	Manometric (13)
pH (urine and whole blood)	Glass electrode, electrometric pH meter Marconi type T511D

The blood pH was corrected to 38°C, employing a correction factor of 0.014 units per degree C. No temperature correction was applied to urine pH.

The bicarbonate concentrations of plasma and urine were derived by insertion of the measured values of pH and total CO_2 in the Henderson-Hasselbalch equation.

Calculation of electrolyte "balance." It was assumed that a steady state of body electrolytes had been obtained when the daily excretion of electrolyte became constant (within the limits of inherent cyclical variation), while the intake remained unvaried. Changes in the total body content of electrolyte during the ingestion of Diamox®, or during recovery from its effects, were calculated as the difference between the observed rate of urinary electrolyte excretion for that day and the control rate.

The term "conservation" applied to electrolytes is used to mean retention of electrolyte with reduction of an existing negative balance. Urinary "hydrogen ion" refers to the sum of urinary ammonium ion and titratable acid, in accordance with current theories of hydrogen ion excretion (14–18).

RESULTS

The changes in urine flow and acid-base composition of the urine for the whole period of observation are shown in Tables I to IV.

I. *Balance Experiments in Normal Subjects*

Changes in electrolyte excretion and urine flow during ingestion of Diamox®

On the first day of ingestion of the drug both subjects showed an increase in flow of urine of approximately 1.2 liters; smaller increments of urine flow also occurred on the next two days. There was a corresponding weight loss, which finally amounted to 2.3 kg. and 1.7 kg., respectively.

Total daily electrolyte excretion rose from 400 to 550 m.Eq. in 'A' and from 310 to 500 m.Eq. in 'B' on the first day; on each day thereafter total electrolyte excretion was not materially different from the control period. The sequence of changes in the excretion of HCO_3^-, H^+, and of K^+, was almost identical in the two subjects. Excretion of bicarbonate was greatly augmented on the first day, but dwindled rapidly and attained normal values by the third day; the cumulative totals of bicarbonate excretion were 163 and 132 m.Eq. Excretion of H^+ was reduced to very low levels on the first day and was almost normal by the third. The cumulative buffer decrement (excess

TABLE I

Acid-base composition of the urine and daily electrolyte excretion during ingestion of Diamox® and during recovery from the drugs effects (Subject 'A')

	Day	pH	Na^+ m.Eq.	K^+ m.Eq.	Mg^{++} m.Eq.	Ca^{++} m.Eq.	NH_4^+ m.Eq.	Titr. Acid m.Eq.	Total "Base" m.Eq.	Cl^- m.Eq.	HPO_4^- m.Eq.	SO_4^{--} m.Eq.	Org. Acid m.Eq.	HCO_3^- m.Eq.	Total "Acid" m.Eq.	Body Wt. Kg.
CONTROL(mean)	1-6	-	180	108	8	17	40	45	398	171	91	74	54	4	394	72.48
Diamox 0.25 g. 6-hrly.	7	7.31, 7.02	258	245	12	18	7	12	552	172	105	79	43	133	532	71.27
	8	6.58, 6.50	165	140	10	17	36	40	408	142	109	69	59	19	398	70.77
	9	6.21, 6.52	144	113	7	16	35	33	348	133	98	64	47	9	351	70.14
	10	6.21, 5.97	119	119	9	20	38	34	339	126	97	67	48	2	340	70.17
Recovery	11	5.01, 4.99	131	72	8	21	61	64	357	155	83	71	50	2	361	70.69
	12	4.99, 5.32	142	44	12	20	114	63	395	185	95	61	48	2	391	71.89
	13	5.15, 5.32	206	50	9	22	96	90	473	222	120	80	61	2	485	72.12

Urine pH values are given for 2 12-hour periods of each day

TABLE II

Daily changes in urinary acid-base excretion during ingestion of Diamox® and during recovery from the drug's effects, Subject 'A'

(Computed from Table I)

DAY	Wt. (Kg)	Water Balance (l) Daily	Water Balance (l) Cumulative	Na^+ m.Eq. Daily	Na^+ m.Eq. Cumulative	K^+ m.Eq. Daily	K^+ m.Eq. Cumulative	NH^+_4 plus Titr. Acid m.Eq. Daily	NH^+_4 plus Titr. Acid m.Eq. Cumulative	Cl^- m.Eq. Daily	Cl^- m.Eq. Cumulative	HCO^-_3 m.Eq. Daily	HCO^-_3 m.Eq. Cumulative	"Other Anions" m.Eq. Cumulative
7	-1.21	+1.22	+1.22	+78	+78	+137	+137	-66	-66	+1	+1	+129	+129	
8	-1.71	+0.60	+1.82	-15	+63	+32	+169	-9	-75	-29	-28	+15	+144	
9	-2.34	+0.39	+2.21	-36	+27	+5	+174	-17	-92	-38	-66	+5	+149	
10	-2.31	+0.06	+2.27	-61	-34	+11	+185	-13	-105	-45	-111	-2	+147	+9
11	-1.88	-0.70	+1.57	-49	-83	-36	+149	+40	-65	-16	-127	-2	+145	
12	-1.39	-0.46	+1.11	-38	-121	-64	+85	+92	+27	+14	-113	-2	+143	
13	-0.36	-0.47	+0.64	+26	-85	-58	+27	+101	+128	+51	-62	-2	+141	+21

+ = Increase
- = Decrease
} of urinary excretion as compared with control value.

HCO_3^- excretion plus reduction of H^+ excretion) was 252 and 234 m.Eq. in 'A' and 'B', respectively, when the drug was withdrawn. In both persons a considerable negative balance of K^+ occurred, amounting finally to 185 and 153 m.Eq.

There were considerable differences in the effect of the drug on sodium excretion in the two individuals. Subject 'A' (Tables I and II) showed a negative balance of sodium only on the first day; thereafter there was a small positive balance for each individual day, and the cumulative balance ultimately became positive. The sodium balance in this subject appeared to be largely dependent on changes in chloride excretion (Figure 1), whereas potassium balance seemed independent of chloride. In subject 'B' there was a large negative balance of sodium on the first day, and a small negative balance on each subsequent individual day, with a final deficit of 196 m.Eq. No material change in chloride excretion occurred in this subject, but a large increment of excretion of "other anions" (phosphate, sulphate and organic acids considered jointly) occurred.

Electrolyte excretion during recovery from the effects of Diamox®

The relevant data are contained in Tables I–IV.

During the recovery periods the urine became strongly but not maximally acid. The excretion of ammonium ion and titratable acid was greatly augmented in 'A', in whom the average daily increment of H^+ excretion was 78 m.Eq. Subject 'B' showed an average H^+ increment of only 45 m.Eq. however, and the urine pH tended to be higher than in 'A'. It may be seen from Tables II and IV that the final deficit of fixed cation (Na^+ plus K^+) was 349 m.Eq. in 'B' and only 151 m.Eq. in 'A'. This fact, coupled with the lower increment of H^+ during recovery in 'B', determined the relative duration of the recovery periods; after six days subject 'B' had repaired the cation deficit by only 55 per cent, whereas three days sufficed for almost complete recovery of K^+ balance in 'A', the net (Na^+ plus K^+) balance at this time being slightly positive in this subject owing to sodium retention.

The different patterns of disturbance of anion excretion observed during the drug period was also evident during the initial stages of recovery.

TABLE III

Acid-base composition of the urine and daily electrolyte excretion during ingestion of Diamox® and during recovery from the drug's effects (Subject 'B')

	Day	pH	Na^+ m.Eq.	K^+ m.Eq.	Mg^{++} m.Eq.	Ca^{++} m.Eq.	NH_4^+ m.Eq.	Titr. Acid. m.Eq.	Total "Base" m.Eq.	Cl^- m.Eq.	HPO_4^- m.Eq.	SO_4^{--} m.Eq.	Org. Acid. m.Eq.	HCO_3^- m.Eq.	Total "Acid" m.Eq.	Body Wt. Kg.
CONTROL(mean)	1-7	-	143	53	7	18	43	45	309	150	73	53	32	2	310	61.24
Diamox 0.25 g 6-hrly.	8	7.41,	303	152	5	18	14	5	497	168	95	54	66	117	500	59.95
	9	6.71,	155	84	8	18	25	36	326	139	63	62	42	14	320	59.55
	10	5.78,	167	76	7	18	35	41	344	142	94	80	27	1	344	59.55
Recovery	11	6.21,	113	65	8	18	63	40	307	145	84	59	32	2	322	59.70
	12	5.00,	74	40	7	18	103	52	294	131	98	51	30	2	312	60.30
	13	5.22,	103	27	5	18	98	50	301	137	82	55	27	2	303	60.55
	14	6.33,	151	25	5	16	90	50	339	174	75	53	34	2	338	60.65
	15	5.43,	153	26	7	18	85	45	334	163	73	34	45	2	317	60.55
	16	5.51,	175	33	5	18	73	47	351	177	70	50	39	2	338	-

Urine pH values are given for 2 12-hour periods of each day

TABLE IV

Daily changes in urinary acid-base excretion during ingestion of Diamox® and during recovery from the drug's effects, Subject 'B'

(Computed from Table III)

	Na^+ m. Eq.		K^+ m. Eq.		NH^+_4 plus Titr. Acid m. Eq.		Cl^- m. Eq.		HCO^-_3 m. Eq.		"Other Anions" m. Eq.
DAY	Daily	Cumulative	Daily	Cumulative	Daily	Cumulative	Daily	Cumulative	Daily	Cumulative	Cumulative
8	+160	+160	+99	+99	-69	-69	+18	+18	+115	+118	
9	+12	+172	+31	+130	-27	-96	-11	+7	+12	+127	
10	+24	+196	+23	+153	-12	-108	-8	-1	-1	+126	+109
11	-30	+166	+12	+165	+15	-93	-5	-6	-		
12	-69	+97	-13	+152	+67	-26	-19	-25	-		
13	-40	+57	-26	+126	+60	+34	-13	-38	-		
14	+8	+65	-28	+98	+52	+86	+24	-14	-		
15	+10	+75	-27	+71	+42	+128	+13	-1	-		
16	+32	+107	-20	+51	+32	+160	+27	+26	-	+126	+152

+ = Increase
- = Decrease
} in urinary excretion as compared with control value

There was a further extension of "other anion" losses in 'B' but none in 'A', while the chloride retention observed in 'A' during the drug's action was temporarily increased during recovery (Tables II and IV).

The electrolyte balances at the end of the drug period are compared with those prevailing at the end of the recovery study, in Figure 2.

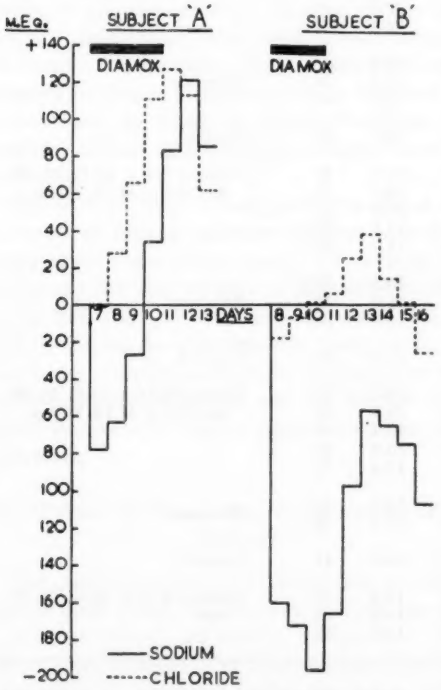

FIG. 1. RELATION OF SODIUM AND CHLORIDE BALANCES DURING ACTION OF DIAMOX® AND DURING RECOVERY OF SUBJECTS 'A' AND 'B'

II. *The Effects of Single Daily Doses of Diamox®*

The changes in urinary excretion of HCO_3^- and H^+ which resulted from ingestion by a normal person of four doses of Diamox® at intervals of one day, are shown in Figure 3. Details of observations in this person and three other normal subjects are shown in Table V.

The initial dose of Diamox® caused an excretion of bicarbonate which ranged from 78 to 97 m.Eq. in the first six hours; during this period the urine pH ranged from 7.50 to 7.70, ammonia excretion was negligible and titratable alkali replaced titratable acid. In the succeeding six hours the urine pH was much reduced, and bicarbonate excretion was less than half that of the first six-

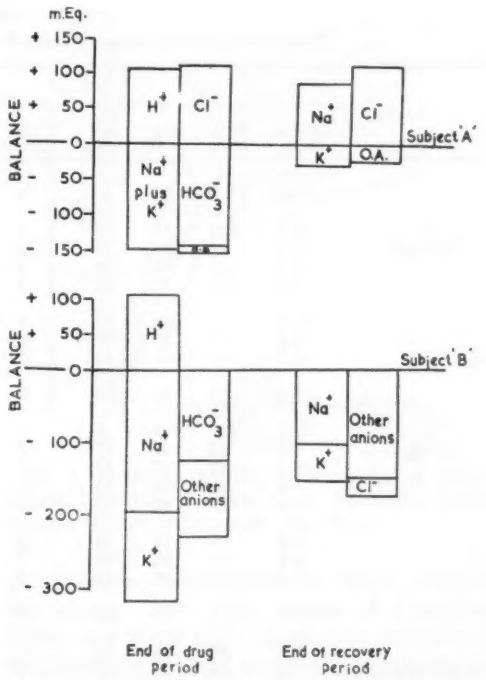

FIG. 2. CHANGES IN BALANCE OF ELECTROLYTES OCCURRING AS A RESULT OF INGESTION OF DIAMOX®, AND DURING RECOVERY FROM THE DRUG'S EFFECTS

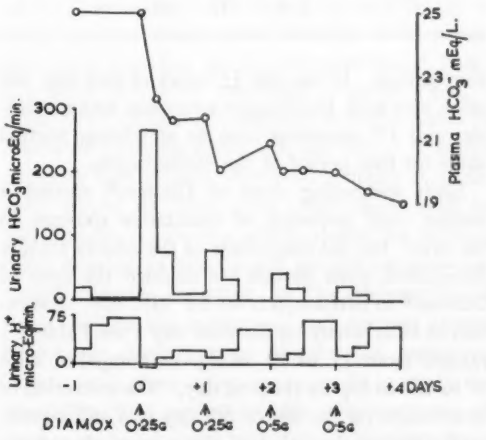

FIG. 3. CHANGES IN PLASMA BICARBONATE AND URINARY EXCRETION OF H^+ DURING ACTION OF SINGLE DAILY DOSES OF DIAMOX®

TABLE V

Effects of single daily doses of Diamox® on urine and plasma of four normal persons

Subject	Urine				Plasma (arterialized)				Doses of Diamox
	Interval (*days*)	HCO_3^- *m. Eq.*	NH_4^+ *m. Eq.*	Titr. acid *m. Eq.*	Time (*days*)	pH	HCO_3^- *m. Eq./L.*	pCO_2 *mm. Hg*	
	(control) −1–0	6	41	41	0	7.40	25.0	42	Control
1. (75 kg.)	0–1	129	11	5	1	7.35	21.8	41	Diamox 0.25 g. at 0, 24 hrs. 0.5 g. at 48, 72 hrs.
	1–2	41	26	25	2	7.36	21.0	38	
	2–3	22	22	36	3	7.38	20.0	35	
	3–4	11	27	28	4	7.37	19.0	34	
	4–5	—	84	62	5	7.40	20.8	35	
	5–6	—	87	49	6	7.41	22.0	36	Recovery
	6–7	—	91	43	7	7.43	23.6	37	
	−1–0	3	41	45	0	7.40	26.3	44	Control
2. (72 kg.)	0–1	120	9	8	1	7.35	21.5	40	Diamox 0.4 g. at 0, 24, 48 hrs. 0.8 g. at 72, 96 hrs.
	1–2	22	25	27	2	7.36	20.1	37	
	2–3	49	17	13	3	7.39	19.0	33	
	3–4	11	31	33	4	7.38	18.3	32	
	4–5	21	30	32	5	7.38	18.9	33	
	5–6	—	73	56	6	7.42	20.3	32	
	6–7	—	64	50	7	7.43	21.9	34	Recovery
	7–8	—	88	51	8	7.43	23.4	36	
	−1–0	7	24	28	0	7.41	21.8	36	Control
3. (61 kg.)	0–1	97	14	11	1	7.37	18.4	33	Diamox 0.25 g. at 0, 24, 48 hrs. 0.5 g. at 72, 96 hrs.
	1–2	24	24	25	2	7.32	16.9	34	
	2–3	25	29	22	3	7.37	16.4	29	
	3–4	25	20	19	4	7.34	16.6	32	
	4–5	13	18	28	5	7.39	17.6	30	
	5–6	—	70	45	6	7.38	20.3	36	
	6–7	—	70	41	7	7.39	20.8	36	Recovery
	−1–0	3	39	39	0	7.39	24.0	41	Control
4. (66 kg.)	0–1	138	20	6	1	7.38	19.0	33	Diamox 0.25 g. at 0, 24, 48 hrs.
	1–2	25	18	10	2	7.40	18.8	31	
	2–3	17	30	10	3	7.38	18.1	32	

hour period. In the last 12 hours of that day, the urine was acid, bicarbonate excretion was negligible, and H^+ excretion rose to an almost normal value for that period of the diurnal cycle.

Each succeeding dose of Diamox® caused a similar daily sequence of qualitative changes in the urine, but the magnitude of the effects rapidly diminished, even though we doubled the dose of Diamox® in two subjects on the third day. Excretion of bicarbonate on the final day (see Table V) ranged from 11 to 21 m.Eq. as compared with 97 to 138 m.Eq. on the first day. The excretion of bicarbonate on the 4th or 5th day was still significantly higher than that of the control day, however, in contrast with the subjects who ingested Diamox® at 6-hour intervals.

The diminishing effect of single daily doses of Diamox® on urinary bicarbonate excretion appeared to be related in a reasonably constant manner in the group of four subjects to concomitant falls of the plasma bicarbonate concentration (Table V). The initial dose of Diamox® caused a fall of plasma bicarbonate concentration which ranged from 3.2 to 5.0 m.Eq. per L. During the second 12-hour ("drug-free") period the plasma bicarbonate rose only very slightly or not at all (Figure 3), and in consequence the next dose was given while the plasma bicarbonate was still depressed. A similar but less pronounced sequence occurred with succeeding doses.

The total H^+ excretion for each drug day was always less than that of the control period, al-

though the deviations grew less each day. The cumulative decrement of H^+ excretion ranged from 50 to 205 m.Eq. while the total buffer decrements varied from 199 to 413 m.Eq., with a mean value of 313 m.Eq.

During recovery from the drug's action the urine became strongly acid, and NH_4^+ excretion was augmented. Subjects 1, 2, and 3 of Table V showed a mean daily increment of 57, 41, and 61 m.Eq. of H^+, respectively. In none of the three had the plasma bicarbonate concentration returned to normal within three days. Interpretation of changes in plasma bicarbonate as an index of recovery from the effects of Diamox® acidosis are rendered difficult by concomitant changes in pCO_2, however.

The data of Table V indicate the extent of disturbance of plasma acid-base balance caused by single daily doses of the drug. The arterial pH fell on the first day by 0.04 or 0.05 units in three of the four subjects; no compensatory fall of arterial pCO_2 occurred during this time, but by the fourth day a reduction of pCO_2 ranging from 6 to 11 mm. Hg had occurred, and had greatly minimized the potential rise of plasma hydrogen concentration.

III. *Effects of Diamox® in Patients with Heart Failure*

The curves of cumulative bicarbonate excretion against time which we observed in eight patients with heart failure, are shown in Figure 4. The curves were of roughly exponential form, and approached a limiting value within the range 150 to 250 m.Eq. The data indicate that the magnitude of the total urinary bicarbonate excretion which occurs before total "resistance" to the action of Diamox® is finally established, is comparable in patients with heart failure to that of normal persons. No measurements of NH_4^+ or titratable acid excretion were made in these subjects; it is probable, however, that the total deficit of buffer caused by the drug was similar to that of normal persons.

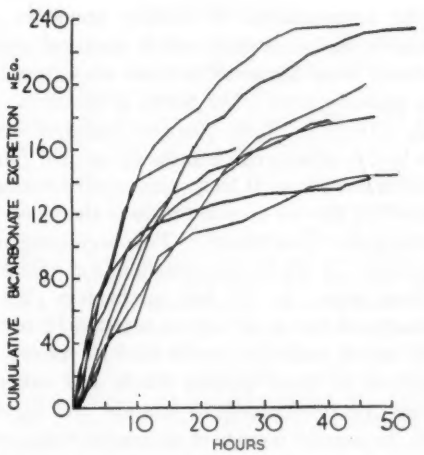

FIG. 4. CUMULATIVE URINARY EXCRETION OF BICARBONATE IN EIGHT PATIENTS WITH CONGESTIVE HEART FAILURE INGESTING DIAMOX® (SEE TEXT)

DISCUSSION

The diminishing effect of Diamox® on urinary bicarbonate excretion has been previously noted by several workers (4, 5, 19), but the nature of the mechanism which limits the drug's action is not entirely clear. The studies of Counihan, Evans, and Milne (20) suggest that lowering of the plasma bicarbonate is the limiting factor, operating through reduction of the load of bicarbonate filtered at the glomeruli. Maren, Wadsworth, Yale, and Alonso (21, 22), however, consider that plasma bicarbonate is "at best a semiquantitative indication of the degree of whole body acidosis following treatment with Diamox®." Irrespective of the ultimate controlling mechanism, it seems probable that Diamox® ceases to cause urinary bicarbonate excretion when a certain total quantity of "bicarbonate-bound base" has been lost from the body. Nevertheless, a number of reports of continued diuretic action of Diamox® have appeared (5, 23, 24). It seems probable that if such sustained diuresis is genuinely due to the drug, then either there must be some mode of recovery which avoids the limiting effect of a loss of bicarbonate ion and permits continued urinary excretion of "bicarbonate-bound base," or else the natriuresis and diuresis cannot be wholly dependent on bicarbonate excretion. The present observations were made to study the possibility that under suitable circumstances of dosage the mechanism and rate of recovery from the metabolic effects of Diamox® would avoid the self-limiting action of the drug.

The augmentation of urinary ammonia and titratable acid excretion which occurred during recovery from Diamox® acidosis also characterizes recovery from other forms of metabolic acidosis (14–18). Both changes represent secretion by the tubule cells into the glomerular filtrate of H^+ derived from H_2CO_3, with parallel regeneration of HCO_3^- for reconstitution of the blood and extracellular bicarbonate. Two well-recognized variations of such compensation for metabolic acidosis exist. In the first mechanism ('a') an exchange of Na^+ or K^+ for the secreted H^+ occurs. This cation exchange would tend to restore any depletion of fixed cations which had occurred during the development of acidosis, and together with the parallel increment of tubular water reabsorption necessary to maintain plasma osmolarity, would result in restoration of both the volume and composition of the extracellular fluid. The balance studies of our two normal subjects suggest that recovery in a normal person is effected predominantly by this mechanism 'a', since approximately 75 per cent of the increment of H^+ excretion during recovery was associated with conservation of fixed cation.

In the second mechanism ('b'), excess H^+ secreted during recovery from acidosis is eliminated in the urine in association with chloride ion withdrawn from the extracellular fluid. Chloride ion withdrawn in this way is replaced by bicarbonate ion derived from H_2CO_3, and "freed" by elimination of the excess H^+. This chloruresis would tend to restore the anion pattern of the plasma; furthermore, no Na^+ or K^+ exchange for H^+ need occur in order to eliminate an excess of H^+ in this instance, and there would, therefore, be no tendency for increased water reabsorption, unless plasma osmolarity had been changed by the original diuresis. Exclusive operation of mechanism 'b' during recovery would allow regeneration of body bicarbonate and thus repetition of the natriuresis and diuretic effects of Diamox® while the extracellular fluid volume was still lower than the pre-Diamox® value. In their balance studies of six subjects, Leaf, Schwartz, and Relman (19) observed an augmented excretion of both ammonium and chloride ions during recovery from the effects of Diamox®, a finding which suggests that mechanism 'b' may at least be partly responsible for recovery from the effects of Diamox® in patients with heart failure.

The present observations were made on normal persons, and it is impossible to draw from them any conclusions as to which qualitative mode of recovery from Diamox® acidosis would be likely to occur in edematous subjects. Certain quantitative aspects of the problem may be discussed in the light of these data, however. The actual time needed for full recovery from the effects of Diamox® will depend on the relative magnitudes of the original buffer depletion and the rate at which excess H^+ can be secreted by the renal tubules during recovery.

The observed daily increments of H^+ excretion ranged from 40 to 80 m.Eq., with a mean of 56 m.Eq. Recovery of the average buffer decrement of 290 m.Eq. in our six normal subjects will thus require 5 to 6 days; the recovery time is highly unlikely to fall below three days, and may be as long as 7 to 9 days.

Similar considerations may be applied to recovery from a solitary dose. The initial dose of Diamox® (0.25 to 0.4 g.) caused a decrement of base which ranged from 117 to 189 m.Eq. (Table V). The mean rate of H^+ increment, 56 m.Eq. per day would thus necessitate, on average, 2 to 4 days for recovery. This finding is in general agreement with Maren, Wadsworth, Yale, and Alonso's (21) statement that "it takes 3 to 5 times longer in the dog to repair a HCO_3^- or base deficit than to create it."

Our observations on the self-limiting action of Diamox® in single daily doses in human subjects, are wholly at variance with the results of Maren, Wadsworth, Yale, and Alonso (21) in dogs. These workers found that Diamox®, in a dosage of 5 to 10 mg. per kg. body weight, did not cause sustained depression of plasma bicarbonate. This was due to the fact that extra acidification during the "drug-free" period after each dose was associated with an H^+ increment sufficient for regeneration of bicarbonate lost in the initial phase of the drug's action, and the plasma bicarbonate was normal when each dose of Diamox® was given. The results already described, were different in all our subjects, and the self-inhibiting effect of Diamox® was apparently determined by the fall of plasma bicarbonate, as described by Counihan, Evans, and Milne (20).

A notable feature of one balance study (subject 'A') was the considerable retention of sodium ion which occurred and the large concomitant positive balance of chloride. It is improbable that this was caused by a fortuitous normal cyclical variation in body salt content as no comparable variation was observed in the 6-day control period. The erratic nature of the changes in urinary chloride excretion caused by Diamox® has also been noted by Maren, Wadsworth, Yale, and Alonso (21). It is probable that a chloruresis may be caused by Diamox® in some circumstances: Schwartz, Relman, and Leaf (24) noted that the large diuresis (and presumed natriuresis) which they observed in some of their edematous subjects, could only be accounted for by chloruresis.

SUMMARY

1. Detailed studies have been made in two normal persons of the changes in urinary electrolyte excretion which occur during ingestion of Diamox®, and during recovery from the drug's effects. It seems probable that recovery of normal subjects is effected by mechanisms similar to those seen in other forms of metabolic acidosis, *i.e.*, exchange of fixed cation for H^+. Alternative mechanisms of recovery exist and the therapeutic implications of the various mechanisms are discussed.

2. The self-limiting effect of Diamox® in single daily doses is described.

3. The total buffer decrement caused by Diamox® (when the drug was ingested to the point where it ceased to cause urinary bicarbonate excretion) ranged from 250 to 400 m.Eq. The rates of urinary H^+ increment observed during recovery from the drug's effects suggest that 5 to 6 days will be required on average for full recovery from a series of doses of Diamox®, and 2 to 4 days for recovery from an initial single dose.

ACKNOWLEDGMENTS

We are grateful to Professor C. H. Stuart-Harris and Dr. C. E. Davies for their encouragement and advice. Valuable technical assistance was given by Mr. A. F. Nicholls and Miss M. S. Greaves. Diamox® was generously supplied by Messrs. Lederle.

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THE DISTRIBUTION OF CHLORMERODRIN (NEOHYDRIN®) IN TISSUES OF THE RAT AND DOG[1]

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No less than three lines of evidence suggest that organic mercurial compounds exert a diuretic action by virtue of their specific accumulation in renal tubular cells. Govaerts (1) showed that a kidney, removed from a donor animal at the peak of mercurial diuresis, maintains a high rate of urine flow when transplanted into a nondiuretic recipient animal. Bartram (2) demonstrated that the injection of a minute dose of a mercurial diuretic into the artery supplying one kidney causes a prolonged diuresis limited to that kidney alone. Immediately following an intravenous injection of a mercurial diuretic Weston, Grossman, Lehman, Ullmann, Halperin, and Leiter (3) observed that the rate of removal of mercury from the arterial blood by the kidney exceeds the rate of excretion of mercury into the urine.

It is now generally agreed that mercurial diuretics block the renal tubular reabsorption of some limited fraction of the sodium and chloride ions filtered through the glomeruli (4). The elimination of this increased urinary load of osmotically active ions obligates the excretion of water, hence induces polyuria and loss of weight (5, 6). It is probable that mercurial diuretics interfere with some enzyme system or systems concerned either directly with the active tubular transport of ions or with the supply of energy to the transport mechanism. Although blockade of succinic dehydrogenase has been implicated in mercurial diuresis (7–10), evidence is by no means so conclusive as to demand general acceptance (11, 12).

The study described here provides quantitative information on the degree to which mercury is concentrated in the kidney and in other tissues following the injection of a mercurial diuretic, both as a function of time and as a function of administered dose. We have, in addition, studied the action of dithiopropanol on the distribution and excretion of mercury. Ease, rapidity, accuracy and sensitivity of analytic methods directed the use of a diuretic tagged with radiomercury (Hg^{203}) and quantified by gamma emission in a well-type scintillation counter. The rat was chosen as the major experimental animal because of its extensive use in recent histochemical and enzymatic studies (7–10) bearing on site and mode of action of mercurial diuretics. Limited data on the dog have been obtained for comparison. Because of the simplicity with which radiomercury can be incorporated, Chlormerodrin, 3-chloromercuri-2-methoxypropylurea (Neohydrin®) has been chosen for study. Although the analytic method employed quantifies mercury, not the diuretic molecule *per se*, this constitutes no serious drawback for two reasons. First, the significant component of the diuretic is no doubt mercury. Second, the carbon-mercury bond is strong and most probably is not ruptured in the body. Thus Weiner and Müller (13) have shown that Mersalyl is stable in the body and is quantitatively excreted in the urine as a cysteine-like sulfhydryl complex, no detectible mercury being eliminated in inorganic form. The same is probably true of Chlormerodrin (Neohydrin®) (14).

METHODS

3-Chloromercuri-2-methoxypropylurea has been synthesized in our laboratory following directions supplied by Dr. H. L. Friedman of the Lakeside Laboratories. It has been tagged with Hg^{203} and during its useful life has exhibited an activity of from 4000 to 800 counts per minute per microgram mercury in a well-type scintillation counter. The diuretic agent, prepared as the hydrochloride, has been neutralized to phenol red and injected deep into the thigh muscles of rats. We have used female albino rats, weighing roughly 200 gm., obtained from the Charles River Farms. Food and water were permitted *ad lib.* until the start of an experiment

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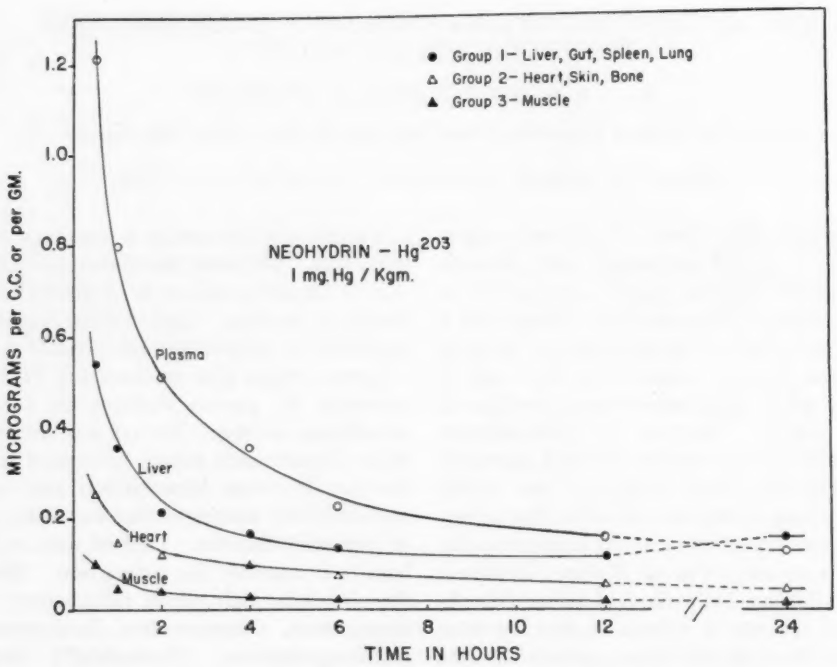

FIG. 1. PLASMA AND TISSUE CONCENTRATIONS OF MERCURY IN RATS FOLLOWING THE INTRAMUSCULAR ADMINISTRATION OF 1.0 MG. Hg^{203} PER KILOGRAM AS CHLORMERODRIN (NEOHYDRIN®)

To avoid confusion, results on only three tissues are included, namely liver, heart, and muscle. Concentrations of mercury in the tissue making up groups 1 and 2 were comparable to those illustrated for liver and heart, respectively. Each datum is a mean value derived from observations on 6 rats.

but not thereafter. Immediately after injection, the rats were placed in individual glass metabolism cages.[3] At the end of the experimental interval (*i.e.*, at ½, 1, 2, 4, 6, 12, or 24 hours), urine was expressed from the bladder by pressure over the lower abdomen, the animal was etherized and blood was rapidly drawn from the inferior vena cava. The animal was then killed and tissue samples were removed, weighed on a torsion balance, and inserted into standard test tubes for counting. One or 2 cc. of plasma, similar volumes of diluted urine and up to 2 grams of tissue were counted. Because we were more interested in total recovery of mercury excreted in the urine than in an estimation of the magnitude of the diuresis, the cage and screen were washed down with some 30 cc. of distilled water and the urine and washings diluted to known volume. One of the two kidneys was decapsulated, weighed, minced and placed in a glass homogenizer with teflon pestle. Sufficient fluid was added to form a 1:10 dilution and the kidney was thoroughly ground. After further dilution, an aliquot was removed for counting. For the most part, sufficient counts were accumulated to ensure an accuracy of from 1 to 5 per cent. For tissues of low activity at 12 and 24 hours, it did not seem worthwhile to prolong counting times to attain this degree of accuracy. Each datum presented is based on the average of individual determinations on six rats. In all, some 72 rats were used.

Data are also presented from two experiments on dogs sacrificed at the peak of diuresis some two hours after an intravenous dose of 1.0 mg. of mercury per kilogram as Chlormerodrin.

[3] These cages were prepared by Dr. Frank Carpenter from 1-liter pyrex beakers by sealing a tube into and drawing down the bottom to form a funnel. A stainless steel screen floor was inserted to prevent contamination of urine with feces.

RESULTS

In Figure 1 are summarized the concentrations of mercury in plasma and in representative tissues other than kidney following the intramuscular injection of roughly 1.0 mg. of mercury per kilogram

TABLE I

Plasma and kidney concentrations of mercury and per cent of the dose bound by the kidneys and excreted in the urine following the intramuscular administration to rats of 1.0 mg. Hg^{203} per kilogram as Chlormerodrin (Neohydrin®)

Number of rats	Time *hours*	Mean body weight *gm.*	Mean weight kidneys *gm.*	Plasma conc. *μ gm./cc.*	Kidney conc. *μ gm./gm.*	Kidney / Plasma	Per cent dose in kidneys	Per cent dose excreted
6	1/2	212	1.494	1.210	42.3	35.0	31.6	0.11
6	1	206	1.504	0.796	55.6	69.9	41.8	0.20
6	2	209	1.436	0.508	75.2	148.0	54.0	0.43
6	4	205	1.458	0.352	97.7	277.0	71.2	1.27
6	6	211	1.480	0.225	108.	480.0	80.0	2.10
6	12	223	1.582	0.154	94.0	610.0	74.3	11.6
6	24	210	1.376	0.124	97.5	786.0	67.1	21.8

of body weight as radio-chlormerodrin.[4] Obviously, absorption from an intramuscular deposit is rapid for the peak plasma concentration was reached within the first half an hour after injection. Thereafter plasma concentration decreased, rapidly for the first six hours and then more slowly over 12 and 24 hours. In all, samples of eight different tissues, other than kidney, were removed from each rat. Since these eight tissues fell into three groups with respect to mercury content, results on only one tissue from each group are included in Figure 1. Thus, liver, gut, spleen, and lung fall into one group exhibiting a moderate concentration of mercury relative to plasma. This group is exemplified by liver. Similarly, a second group, including heart, skin, and bone, is exemplified by heart. Skeletal muscle stood alone in exhibiting a very low concentration of mercury. In general, all tissue concentrations other than kidney, varied in parallel with plasma concentration. Only in the case of liver at 24 hrs. did the tissue concentration definitely exceed the plasma concentration. Probably the most variable tissue of all was gut. The sample, which included duodenum and jejunum, was variably contaminated with bile, although the gut was stripped to remove its contents prior to weighing and counting.

In Table I are summarized the data on the renal uptake and excretion of the mercury of Chlormerodrin from those same experiments presented in Figure 1. Of special interest are the kidney concentrations, expressed in micrograms per gm. of tissue, the kidney/plasma concentration ratios, and the per cent of the dose bound by the kidneys and excreted in the urine. It is evident that within a half-hour after the injection of 1 mg. per kilo of mercury as Chlormerodrin, 32 per cent of the dose was taken up by the kidneys at a concentration of 42 micrograms per gm. tissue. The kidney/plasma concentration ratio was 35. From 4 to 12 hours some 70 to 80 per cent of the dose was fixed in the kidneys at a concentration of approximately 100 micrograms per gm. of tissue.

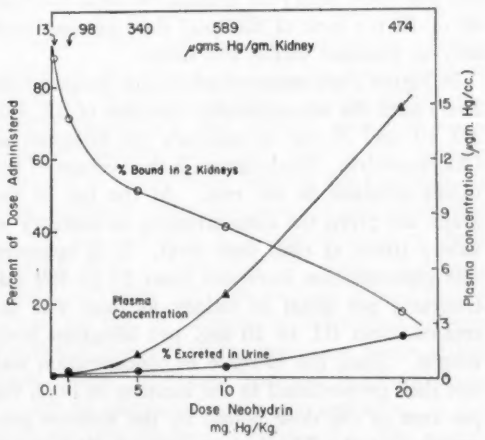

FIG. 2. RELATIONSHIPS AMONG PLASMA MERCURY CONCENTRATION, THE PER CENT OF THE MERCURY BOUND BY THE KIDNEYS, AND THE PER CENT OF THE MERCURY EXCRETED IN THE URINE FOUR HOURS AFTER THE INTRAMUSCULAR INJECTION IN RATS OF 0.1, 1.0, 5.0, 10.0 OR 20.0 MG. Hg^{203} PER KILOGRAM AS CHLORMERODRIN (NEOHYDRIN®)

At the top of the chart, absolute concentrations of mercury are given in terms of micrograms per gram of kidney. Each datum is a mean value derived from observations on 6 rats.

[4] 1.83 mg. of Chlormerodrin contains 1.0 mg. of mercury. Each rat received 200 micrograms of mercury and since the weight of the rats used in this group of experiments averaged 211 gm., the dose administered was slightly less than 1.0 mg. of mercury per kilogram, the usual therapeutic dose of most mercurial diuretics in man.

TABLE II

Effects of BAL (dithiopropanol) on renal uptake and excretion of mercury by the rat *

Condition	Number of rats	Mean body weight gm.	Plasma conc. μ gm./cc.	Kidney conc. μ gm./gm.	Kidney / Plasma	Per cent dose in kidneys	Per cent dose excreted
Diuretic only	6	205	0.352	97.7	277	71.2	1.27
Diuretic plus BAL	6	221	0.549	27.7	50	20.0	27.2

* Each rat received 1.0 mg. Hg^{203} per kilogram as Chlormerodrin (Neohydrin®) intramuscularly. Half of the rats received five times a molar equivalent of 10 per cent BAL in oil intramuscularly.

This is equivalent to a concentration of 5×10^{-4} molar mercury. Mercury *in vitro* in such concentration is capable of blocking a number of enzyme systems (15).

The kidney/plasma concentration ratio steadily rose throughout the 24-hour period. However, it should be noted that from the fourth to the twenty-fourth hour, the increase in ratio was due to the drop in plasma concentration, for the kidney concentration remained essentially constant. Urinary excretion was negligible for the first six hours and even after 24 hours only 22 per cent of the dose had been eliminated. In this respect, the rat differs radically from both man (16, 17) and dog (*vide infra*) for in these forms as much as 40 or 50 per cent of the total dose administered may be excreted within two hours.

In Figure 2 are summarized results obtained four hours after the intramuscular injection of 0.1, 1.0, 5.0, 10, and 20 mg. of mercury per kilogram as Chlormerodrin. Each datum is the average of the values obtained in six rats. At the top of the graph are given the concentrations of mercury in kidney tissue at each dose level. It is apparent that concentration increased from 13 to 589 micrograms per gram of kidney as dose was increased from 0.1 to 10 mg. per kilogram body weight. Since the increase in concentration was less than proportional to the increase in dose, the per cent of the dose bound by the kidneys progressively fell. The largest dose of 20 mg. per kilogram was frankly toxic and the animals appeared in poor condition. One was anuric. The fact that the kidneys bound less mercury at this dose level than at 10 mg. per kilogram is no doubt a reflection of their poor physiologic state.

Dithiopropanol (BAL) is an effective antidote in mercury poisoning (18) and has been shown to abolish mercurial diuresis (19). Both actions are presumably due to the formation of an inactive mercaptide, in the one instance, with the two free valences of inorganic mercury, in the other instance, with the single free valence of the organically combined metal. In Table II are summarized the effects of BAL on the binding and excretion of mercury by the kidneys. In the first group of six rats, the diuretic alone was administered in the amount of 1 mg. of mercury per kilogram. In the second group of six rats, the same dose of diuretic was given, but simultaneously five times a molar equivalent of 10 per cent BAL in oil was injected into the opposite thigh. Four hours later, both groups of rats were sacrificed. As a consequence of treatment with BAL, only 20 per cent of the administered dose of mercury was bound in the kidneys at a concentration of 27.7 micrograms per gm. In contrast, in the untreated animals, 71.2 per cent of the dose was bound in the kidneys at a concentration of 97.7 micrograms per gm. The BAL treated animals excreted 27.2 per cent of the administered dose in four hours,

TABLE III

Effects of BAL (dithiopropanol) on the uptake of mercury by liver and muscle of rats *

	Number of rats	Mean body weight gm.	Plasma conc. μ gm./cc.	Liver conc. μ gm./gm.	Liver / Plasma	Muscle conc. μ gm./gm.	Muscle / Plasma
Diuretic only	6	205	0.352	0.165	0.47	0.028	0.08
Diuretic plus BAL	6	221	0.549	0.393	0.72	0.170	0.31

* Data from same experiments presented in Table II.

whereas the untreated animals excreted only 1.27 per cent.

In Table III are summarized the effects of BAL on the distribution of mercury in liver and muscle of rats. These data are from the same experiments summarized in Table II. A variety of tissues were studied but since all behaved in a qualitatively similar fashion, presentation of data will be limited to these two. The plasma concentration of mercury was higher in the BAL treated animals than in those receiving the diuretic only. Similarly, the liver and muscle concentrations were higher in the BAL treated group. However, tissue concentrations increased more than plasma concentration. Accordingly the liver/plasma and the muscle/plasma ratios rose in consequence of BAL treatment. It is obvious that the effect of BAL on the accumulation of mercury in kidney tissue is exactly opposite to its effect on all other tissues. In kidney, BAL reduces the tissue accumulation of mercury. In all other tissues studied, namely liver, spleen, lung, gut, heart, skin, bone, and muscle, BAL increases the tissue accumulation of mercury. Perhaps it would be better to say that BAL increases the tissue penetration of mercury.

In Table IV are summarized results obtained in two dogs in which a dose of 1 mg. of mercury as Chlormerodrin per kilogram was administered intravenously.[5] Two hours later the animals were sacrificed. Several differences of a quantitative nature between the rat and the dog as well as obvious qualitative similarities are evident from an inspection of this table. Thus, 40 per cent of the administered dose of Chlormerodrin was excreted in two hours by the dog. Less than 1 per cent was excreted by the rat during the same time interval. In the dog, several tissues other than the kidney, namely liver, spleen and adrenal, may concentrate mercury relative to plasma, *i.e.*, exhibit a tissue/plasma ratio greater than 1.0. In the dog, mercury is highly concentrated in the outermost layer of the renal cortex, whereas in the papillary part of the medulla it is concentrated only moderately with respect to the plasma and indeed is less concentrated than it is in the urine excreted just prior to sacrifice.

[5] We acknowledge our indebtedness for these data to two groups of Second Year Medical students who performed these experiments under our supervision in the course of Project Teaching in Physiology.

TABLE IV

Plasma and tissue concentrations and rates of excretion of mercury in two dogs two hours after the intravenous injection of 1.0 mg. Hg^{203} per kilogram as Chlormerodrin (Neohydrin®)

Tissue	Dog A		Dog B	
	μ gm./gm. or/cc.	Tissue / Plasma	μ gm./gm. or/cc.	Tissue / Plasma
Plasma	0.91	—	1.02	—
Kidney				
Outer cortex	163.	179.	131.	128.
Medullary papilla	2.17	2.38	3.38	3.31
Liver	2.82	3.10	2.30	2.26
Spleen	1.93	2.12	0.86	0.84
Intestine	0.71	0.78	—	—
Adrenal	0.51	0.56	1.66	1.63
Heart	0.27	0.30	0.29	0.28
Muscle	0.16	0.18	0.11	0.11
Excretion in 2 hours	40.3% of dose		40.8% of dose	

DISCUSSION

The most impressive feature of our observations is that in both rat and dog the mercury of Chlormerodrin is highly concentrated in the kidneys relative to the blood plasma and in comparison with any other tissue. While others (20, 21) have observed some concentration of mercury in the kidneys of man and the rabbit following organic mercurial diuretics, none made his observations at times of maximum uptake. If one makes the logical assumption that organic mercurial compounds combined reversibly with certain cellular enzymes to render them inactive, the predominant renal action of these compounds is understandable on the basis of their high concentration in renal tubular cells. According to Greif, Sullivan, Jacobs, and Pitts (22), the mercury is most highly bound per mg. of nitrogen by the soluble protein fraction of the cells of the outermost layers of the renal cortex. However, mercury is firmly fixed, though in somewhat lesser concentration, in the mitochondrial fraction of these cells. The nature of the proteins or the organelles of renal cortical cells which bind mercury and the reasons why they, rather than the proteins and organelles of other tissues, preferentially bind mercury are factors of significance to an understanding of diuretic mechanism. Unfortunately we can shed no light on them at the moment.

It is tempting to postulate that the delay in onset of diuresis following administration of an organic

mercurial diuretic is related to the time required to build up some critical concentration of mercury within the tubular cells. Diuresis might then be sustained for a period of high cell content of mercury and diminish as excretion of mercury began to outstrip cell uptake. In the rat, the slow excretion and the sustained high cell content of mercury would argue for a prolonged diuretic action. In fact, according to Fawaz and Fawaz (11), mercurial diuresis in the rat is slow in onset and prolonged in duration. In contrast, the brisk onset of diuresis in the dog may be correlated with very rapid uptake of mercury and the relatively brief duration of diuretic action may be correlated with rapid excretion of mercury (23). We must however, temper our enthusiasm for this thesis by noting that the rat still retains a major part of the mercury in the kidneys after 24 hours, yet according to Fawaz and Fawaz (11), is not diuretic at that time. In both rat and dog, the inhibition of diuresis by BAL is associated with reduced renal binding and more rapid excretion of mercury.

Both Meralluride (24) and Chlormerodrin (25) are known to be highly bound to plasma proteins. Hence only insignificant traces of these diuretics enter the urine in the glomerular filtrate. Weiner and Müller (13) have shown that Mersalyl and Chlormerodrin as well (14) are excreted in the urine of the dog in combination with some short chain sulfhydryl compound such as cysteine and postulate that this complex is a detoxification product. If the uptake and concentration of mercury within the tubular cell is associated with diuresis, then the combining of the diuretic with cysteine and the secretion of the complex in the urine may be associated with the restoration of normal reabsorptive function of the tubular cells. It is possible that the cysteine metabolism of the rat kidney is much less active than that of the dog kidney and hence that the excretion of mercury is proportionately slower. The administration of BAL to either the rat or dog provides a large excess of sulfhydryl groups with which to complex the diuretic. Accordingly, renal concentration is reduced and rate of excretion increased. However, BAL must have some additional action, for it increased the concentration of mercury in all tissues other than kidney. It might do so by reducing binding to plasma proteins and by increasing diffusibility across cell membranes, perhaps by increasing lipid solubility.

Adam (26) has recently shown that the kidney of the rabbit concentrates mercury administered as the bichloride salt. Fitzsimmons and Kozelko (27), have made similar observations in rhesus monkeys. Both groups have shown that BAL reduces renal binding, Adam emphasizing great enhancement of urinary excretion by the dithiol, Fitzsimmons and Kozelko pointing out that it increases penetration of all tissues other than kidney by mercury.

CONCLUSIONS

1. Following the intramuscular administration of 1 mg. of mercury per kilogram as Chlormerodrin (Neohydrin®) to rats, renal uptake of mercury reached a peak within six hours. Some 80 per cent of the administered dose was bound by the two kidneys at a concentration of approximately 100 micrograms of mercury per gm. of tissue.

2. The concentration of mercury in the kidneys of rats was sustained at a high level for more than 24 hours. Excretion was slow; only 21 per cent of a dose of 1 mg. per kilogram was eliminated in the urine in 24 hours.

3. Increasing the dose of Chlormerodrin from 0.1 to 10.0 mg. of mercury per kilogram failed to saturate the renal mechanism which binds mercury, although the per cent of the administered dose bound by the kidneys fell progressively.

4. No tissue studied bound mercury in a concentration even vaguely approaching that of the kidney.

5. Dithiopropanol (BAL) administered simultaneously with Chlormerodrin reduced the renal binding of mercury and augmented the rate of excretion of mercury. BAL likewise increased the diffusibility of mercury and caused an increase in the content of the metal in all tissues other than kidney.

6. The kidney of the dog similarly binds mercury in high concentration. Two hours after a dose of 1 mg. of mercury per kilogram as Chlormerodrin and at the peak of diuresis, the outer cortex of the kidney contained 130 to 160 micrograms of mercury per gm. The papillary portion of the medulla contained relatively small amounts of mercury. The dog differs from the rat in having somewhat higher concentrations of mercury in

liver and spleen and in excreting the mercury very rapidly in the urine.

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DISTRIBUTION OF RADIOMERCURY ADMINISTERED AS LABELLED CHLORMERODRIN (NEOHYDRIN®) IN THE KIDNEYS OF RATS AND DOGS[1]

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Although the mechanism by which mercurial diuretics exert their effect is still obscure, the experiments of Bartram (1) strongly favor direct renal action. Further studies have shown that following administration of such compounds to humans and to animals a higher concentration of mercury is found in the kidney than in the other tissues examined, (2, 3) and it appears that renal binding precedes the onset of diuresis (4, 5). A number of such investigations have been carried out with the use of diuretics labelled with Hg^{203} (2, 3), and this isotope administered as $HgCl_2$ has been found by radioautographic means in high concentration in the renal cortex of rats (6).

The experiments to be reported consist of a detailed study of the gross and subcellular localization of radiomercury administered as labelled Neohydrin® (7) in the kidneys of rats. The distribution was also examined in the kidneys of dogs sacrificed at the time of maximum diuresis.

PART I. RAT TISSUE STUDIES

Method

Two hundred gram female albino rats received intramuscular injections of Neohydrin® labelled with radiomercury and were sacrificed by exsanguination under light ether anesthesia. The kidneys were removed, decapsulated, and weighed, and the tissue so obtained was minced with scissors. The mince was ground in cold 0.25 M sucrose by means of a motor-driven homogenizer with "Teflon" pestle, and sufficient sucrose was added to make a 10 per cent w/v suspension. Portions of this material were subjected to differential centrifugation by modifications of the method of Schneider (8).

The "nuclear" fraction was isolated by centrifugation at 600 × g for 10 minutes in an I.E.C. centrifuge with No. 233 head. The precipitate from this step was twice resuspended and recentrifuged in 0.25 M sucrose, as mentioned above. The final residue, resuspended in sucrose, constituted the "nuclear" fraction.

The pooled supernatant fluids from the nuclear isolation and washes were centrifuged at 25,000 × g for one hour in a Servall SS angle centrifuge. The pellet from this operation was resuspended in sucrose and again centrifuged at 25,000 × g for one hour. The resuspended precipitate constituted the "granular" fraction. In certain experiments, "large granules" were isolated by centrifugation of the supernatant fluid from the nuclear isolation at 8500 × g for ten minutes. The supernatant fluid from this step was centrifuged at 25,000 × g for one hour to yield a "small granule" pellet.

The fluid which remains after removal of the solids sedimentable at 25,000 × g for one hour is the "soluble" fraction. In a few instances only "precipitate" and "soluble" are mentioned. These terms refer to the sediment and the supernatant fluid from a single centrifugation of 10 per cent suspension at 25,000 × g for one hour.

All separation operations were carried out at 4°C. The pellets were resuspended in a tissue grinder for sucrose washings. Fractions were carefully checked by phase microscopy. Radioactivity was measured in a Nuclear Instrument well-type scintillation counter with No. 163 scaling unit. Nitrogens were determined by means of a titrimetric semimicro Kjeldahl method using a mercury catalyst.

Results

Table I and Figure 1 summarize the relationship between the mercury and nitrogen content of the kidney fractions obtained from rats. Despite variation both in the amount of mercurial diuretic administered and the time of sacrifice of the animal, the largest amount of mercury per milligram of nitrogen was consistently found in the "soluble" fraction. The ratio of the amount of mercury in the soluble fraction per milligram of nitrogen to the amount of mercury in the "total" suspension per milligram, here designated as the S/T ratio, remains quite constant at all dosage levels tested.

In separate experiments, "soluble" fraction from

[1] This investigation was supported by a research grant from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Bethesda, Md.

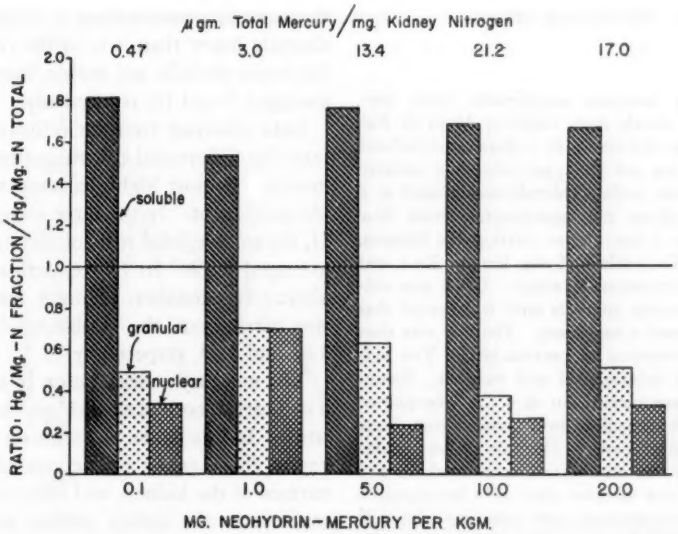

FIG. 1. RELATIONSHIP BETWEEN MERCURY CONCENTRATION IN KIDNEY FRACTIONS AND WHOLE KIDNEY FOLLOWING ADMINISTRATION OF INCREASING AMOUNTS OF CHLORMERODRIN

the kidneys of animals which had received 10 mg. radiomercury per kilo was centrifuged at 105,000 × g for one hour in the Spinco Model G preparative centrifuge with negligible sedimentation of radioactivity. Preliminary experiments indicate that the mercury remains firmly bound to the "soluble" fraction during dialysis against pH 8.6 barbital buffer 0.1 ionic strength, and that most of it is precipitated when this kidney fraction is brought to half saturation with ammonium sulfate.

In all but one of the series of experiments the amount of mercury bound to the "granular" fraction, based on nitrogen, is higher than that in the "nuclear" fraction. An experiment was performed in which the "granular fraction" was isolated from the kidneys four hours following the administration of 1 mg. radiomercury as Neohydrin® to rats. This fraction was repeatedly washed with sucrose. The once-washed starting material contained 1.78 micrograms mercury per milligram nitrogen, and the figures for four washes and seven washes were respectively 1.74 and 1.80 micrograms per milligram nitrogen.

TABLE I

Micrograms radiomercury per milligram nitrogen in rat kidney fractions

Dose mg./K	Sacrifice time	Number animals	"Total"	"Nuclei"	Granules	Soluble	Recovery		Ratio S/T
							Nitrogen	Mercury	
0.1	4	3	.47	.16	.23	.85	97.5	96.2	1.81
1	4	1	4.2	2.7	4.3	6.4	92.5	96.5	1.52
1	4	2	3.0	2.1	2.1	4.6	95	89.9	1.53
5	4	6	13.4	3.2	8.2	23.6	102	96.5	1.76
10	4	6	21.2	5.8	8.1	35.6	101.4	94.5	1.68
20	4	6	17.0	5.6	8.7	28.3	97.6	93.5	1.67
				Precipitate					
1	1	1	3.3		2.5	4.9	93.5	93	1.48
1	4	1	3.6		2.6	6.4	100	102	1.78

PART II. DOG TISSUE STUDIES

Method

Three technically adequate experiments were performed on mongrel female dogs weighing 14 to 21 Kg. After anesthesia was induced with sodium pentobarbital (not more than 30 mg. per Kg.), an indwelling catheter was inserted. Isotonic sodium chloride was infused at 5 ml. per min. throughout the experiment. Urine flow became stable 1½ to 2 hours after starting the infusion. At this time Hg^{203}-Neohydrin® (1 mg. Hg per Kg.) was given as a single intravenous injection. Urine was collected at 10 to 15-minute intervals until it appeared that urine flow had attained a maximum. The dog was then sacrificed with an overdose of pentobarbital. The kidneys were removed, decapsulated and weighed. Subsequent procedures were carried out at 4°C. One-quarter of one kidney was homogenized in a motor-driven tissue grinder using a "Teflon" pestle. The bulk of the remaining tissue was separated into cortex, outer medulla, and inner medulla.[2] These samples were also homogenized by machine. All homogenates were made up with 0.25 M sucrose to a suspension containing 10 grams of tissue per 100 ml. of solution.

The schema of differential centrifugation employed with the dog kidneys was in general similar to that described for the rat kidney fractionations.

For the study of the intrarenal distribution of radiomercury a kidney was sectioned parasagittally and a cylindrical core was obtained by passing a sharp corkborer through the kidney along an axis normal to the surface and traversing a medullary pyramid. This core was cut free-hand into 10 to 12 sections which were transferred to tared counting tubes for the determination of Hg^{203} activity. In one core nitrogen was determined and a small fragment of each section fixed and stained for histologic study.

Results

In Table II are presented data depicting the distribution of Hg^{203} in various regions of the kidney, as well as its distribution in subcellular fractions of kidney cortex. It is apparent that the mercury concentration in the cortex is greater than its average concentration in the whole kidney. The cortex/whole kidney concentration ratio averages 1.7. Similar data on the distribution of mercury in outer and inner medulla indicate that the mercury concentration in these regions is consistently lower than it is in the cortex. The cortex/outer medulla and cortex/inner medulla ratios averaged 9 and 16, respectively.

Data obtained from subcellular fractions separated by differential centrifugation reveal that the mercury is most highly concentrated in the soluble component. In the three experiments in Table II, the soluble/total ratio of mercury concentration averaged 1.59. In two experiments in which a similar fractionation of outer and inner medulla was carried out, the soluble/total ratios averaged 1.67 and 1.54, respectively.

The mercury concentration in sections cut from a cylindrical core of dog kidney is depicted in Figure 2. In this figure, the units on the abscissa represent the number of sections starting from the surface of the kidney, and thus correspond to distance from the kidney surface measured in arbitrary units. The region from which each section was taken, as judged by gross inspection and histologic examination, is indicated at the top of the figure. It is clear that the mercury is most highly concentrated in the outer cortex. The concentration falls very rapidly in the outer region of the medulla, and attains its lowest levels—scarcely higher than that of plasma—in the inner medulla. The mercury concentration in plasma samples drawn just prior to removal of the kidneys averaged 0.74 micrograms per milliliter.

DISCUSSION

The above results are an extension of the work of others who have demonstrated that the mercury in a mercurial diuretic is found in highest concentration in the kidney (2, 3).

The progressive decrease of mercury concentration from a maximum in the outer cortex to a minimum value in the medullary papilla is in accord with the radiographic studies of Lippman, Finkle, and Gillette (6) previously cited. The affinity of mercury for sulfhydryl groups is well established (9); it is therefore not surprising that the intrarenal distribution of mercury observed in our experiments correlates reasonably well with the distribution of protein-bound sulfhydryl groups established by histochemical studies (10). However, such groups are present in comparable concentration in other tissues (myocardium, skeletal

[2] In this experiment the outermost zone of the kidney was considered to be "cortex." This tissue was dark red and granular in appearance and contained some medullary rays. The "outer medulla" consisted of pale striated medullary pyramids, but also included much cortical tissue occurring between these pyramids in columns. The "inner medulla" consisted largely of pale fibrous papillary tissue.

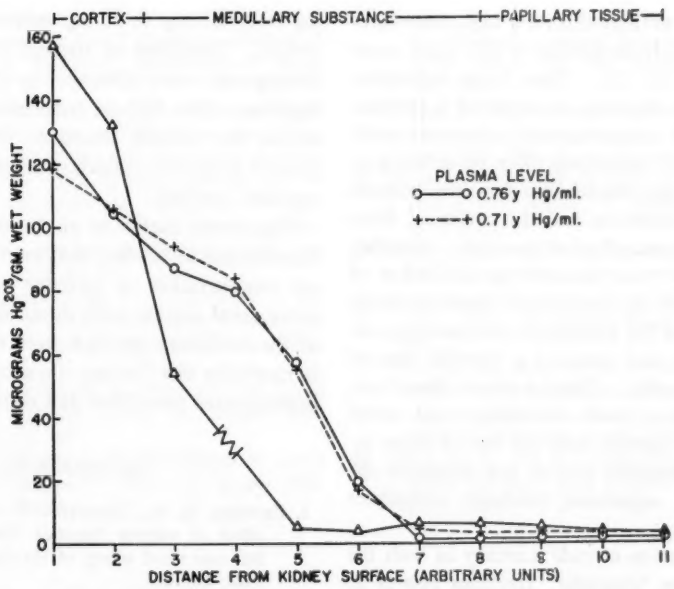

FIG. 2. RELATION BETWEEN MERCURY CONCENTRATION AND DISTANCE FROM KIDNEY SURFACE OF SERIAL SEGMENTS OF RENAL TISSUE

muscle) which bind much less radiomercury; hence, factors unique to the kidney must be involved.

The results of the studies of the apparent intracellular distribution of mercury can be interpreted in several ways. The large amount of mercury in the soluble fraction is most readily explained by the presence in this fraction of mercury-binding proteins in high concentration. The relatively constant S/T ratio over a wide dosage range suggests that the capacity of these proteins to bind mercury has not been exceeded with the largest amount of diuretic employed. The observed decrease in mercury content of the kidneys following the administration of amounts of the diuretic in excess of 10 mg. per kilo suggests that the limiting factors are related to toxic effects, possibly cardiovascular, to the animal rather than to the inability of these proteins to bind additional mercury. In our studies there is no evidence for or against concentration of the mercury in the soluble fraction by means of an active process. In the interpretation of these findings caution must be exercised in relating to *in vivo* conditions the results of localization studies carried out on diluted suspensions of broken cells.

Mercurial diuresis is presumed to occur through inhibition of reabsorption of water and electrolytes in the convoluted tubules, although the problem of whether the locus of action is primarily on the proximal or distal portion has not yet been settled (11). The tubular structures in question predominate in the regions of highest radiomercury concentration. Histochemical techniques have re-

TABLE II

Micrograms radiomercury per milligram nitrogen in fractions obtained from kidneys of dogs sacrificed during diuresis from 1 mg. Hg^{203} per kilo given as Neohydrin® intravenously

Sacrifice time *min.*	Whole kidney	Cortex			Recovery		
		Total	Precipitate	Soluble	Nitrogen	Mercury	S/T ratio
105	1.9	2.9	1.4	4.6	100	96.3	1.57
120	3.26	5.5	2.8	8.9	98.2	96.2	1.62
120	3.54	6.4	3.0	10.7	102	101	1.57

cently been used to demonstrate a high concentration of succinic dehydrogenase in this same outer cortical region (12, 13). Since toxic concentrations of mercurial diuretics are required to produce visible changes in enzyme activity, currently available histochemical techniques offer no solution to the question of the relationship between succinic dehydrogenase inhibition and the action of these substances in pharmacological amounts. Handley and Lavik (14) have reported an inhibition of succinoxidase activity determined manometrically in homogenates of the kidneys of rats and dogs after these animals had received a diuretic dose of an organic mercurial. Other workers have been unable to produce such inhibition, and could neither produce diuresis with the use of other inhibitors of this enzyme system nor implicate the system through sequential blocking techniques (15, 16).

The demonstration of radiomercury in both the "soluble" and the "granular" fractions relates to this problem only in that mercury seems firmly bound to the structures which are believed to be important in the process of ion transport (17, 18). Succinoxidase is only one of the many enzyme systems associated with the mitochondria included in our "granular" fraction (19).

Cohen, DeGroot, and Weber (20, 21) have recently demonstrated that diuretic amounts of organic mercurials inhibit both the formation and the breakdown of energy-rich phosphate compounds in slices of rat kidney. Slices were used because this inhibition could not be shown with preparations of mitochondria washed with potassium chloride, and the suggestion was made that washing removed the mercurial diuretic from these structures (22). Our results indicate that the mercury is firmly bound to granules washed with sucrose, and if such a preparation should also fail to demonstrate inhibition, some other explanation must be given for his findings. It seems possible that the high concentration of mercury in the "soluble" fraction, which would be removed during the isolation of mitochondria, may be important in the inhibition process.

SUMMARY

Chlormerodrin labelled with Hg^{203} was administered intramuscularly to rats in amounts ranging from 0.1 mg. to 20 mg. mercury per kilo body weight. Fractions of sucrose suspended kidney homogenate were obtained by differential centrifugation. The highest concentration of mercury was in the "soluble" fraction. Mercury was also present in granules which could not be removed by repeated washing.

Dogs were sacrificed at the time of maximum diuresis due to labelled chlormerodrin. The highest concentration of mercury was found in the outer renal cortex, with diminishing radioactivity as the medullary papillae were approached. The intracellular distribution of mercury in dog kidney homogenates resembled that of rat kidney.

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STUDIES ON THE METABOLISM OF PLASMA PROTEINS IN THE NEPHROTIC SYNDROME. I. ALBUMIN, γ-GLOBULIN AND IRON-BINDING GLOBULIN[1]

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Although there is unanimity of opinion that the nephrotic syndrome is characterized by proteinuria, hypoproteinemia, edema and hyperlipemia, there is considerably less agreement as to the mechanisms which result in these findings. It is now generally held that the proteinuria found in this disease is related directly to the renal lesion (1) and is not attributable to the excretion of abnormal plasma proteins (2). The cause of the severe hypoproteinemia in this disease, however, is a more controversial subject. The association of proteinuria and hypoproteinemia as cause and effect has been postulated by a number of investigators (1–4) and the occasionally prodigious amounts of plasma protein excreted by children with the nephrotic syndrome makes the relationship attractive. This concept, however, is not universally accepted (5), and it has long been suggested that the degree of hypoproteinemia may not depend upon the severity of proteinuria alone (6).

This study was undertaken in an effort to determine the factors responsible for hypoproteinemia in children with the nephrotic syndrome. Since the concentration of a given plasma protein in the circulation is dependent upon its rate of synthesis, its rate of loss from the body whether by catabolism or excretion or both, and its distribution within the body, an attempt was made to estimate these quantities for albumin, γ-globulin and iron-binding globulin during various stages of this disease.

METHODS

Patients: Six children with the nephrotic syndrome were selected for study. The pertinent medical data are summarized in Table I. It will be noted that three recognizable stages of the disease were represented: 1) Almost complete remission in Group I (one child with residual proteinuria, but without hypoproteinemia, edema, ascites or hyperlipemia); 2) partial remission in Group II (two children with proteinuria, hypoproteinemia and hyperlipemia but only minimal *intermittent* local edema and no recognizable ascites); 3) full-blown disease in Group III (three children with proteinuria, hypoproteinemia, anasarca, ascites and hyperlipemia).

These children were fully ambulatory throughout the study, which took place during the summer months. No evidence of infection was observed in these children during the course of the investigation.

Method of study: Each of the six patients was first given radio-iodinated albumin and, when the radiation in urine and plasma had fallen to background level, radio-iodinated γ-globulin was injected. After the latter, when background level was again attained in urine and plasma, three of the children were given radio-iodinated iron-binding globulin. The proteins were injected intravenously; the specific activities of these protein preparations were such that no more than 1 mg. of iodoalbumin, 0.5 mg. of iodinated γ-globulin or 0.2 mg. of iodinated iron-binding globulin was injected per patient. The maximum radiation employed in any given injection was 1.5 microcuries per kilogram body weight. Beginning 24 hours prior to the administration of radio-iodinated albumin and continuing throughout the period of study, the children were given 10 drops of Lugol's solution three times a day.

Radio-iodinated plasma proteins: Each lot of radio-iodinated plasma protein used in this study was tested for free and loosely bound radio-iodide by dialysis, by precipitation of protein in 10 per cent trichloracetic acid in the presence of suitable carrier protein, and by precipitation of specific protein in the zone of antibody excess with specific rabbit antiserum (7). Control studies indicated that unbound or loosely bound radio-iodide could be completely recovered in the dialysate or supernatants of these procedures. The three methods gave similar results.

One preparation of iodinated human plasma albumin obtained from Abbott Laboratories, No. 806-169-50, was considered satisfactory and was used in each of the six patients; less than 2 per cent of the radio-iodide in this preparation was unbound according to the above methods. Using the quantitative precipitin reaction, the iodoalbumin

[1] Supported by grants from the National Institutes of Health, U. S. Public Health Service (A-251), from the Playtex Park Research Institute, and from the U. S. Atomic Energy Commission.

TABLE I

Clinical and laboratory data during study period

Patient	Age (yrs.)	Weight* (Kg.)	Height (cm.)	Urea nitrogen (mg. %)	Serum cholesterol (mg. %)	Red blood cells (mill./c. mm.)	Time since onset of illness (mos.)	Ascites	Edema	Gross proteinuria (gm./24 hr.)
Group I										
R. S. B.	5–7/12	19.4–19.8	105	30	243–275	3.81–4.96	44†	0 Disappeared 12 mos. ago	0	1.0–1.6
Group II										
A. B.	6–7/12	24.9–26.0	122	11	725–752	4.97–5.56	44	0 Disappeared 6 mos. ago	± (ankles)	1.6–2.5
E. T.	6–6/12	23.4–23.5	119	34	1218–1318	2.91–3.33	56	0 Disappeared 10 mos. ago	± (orbital)	9.2–9.6
Group III										
K. S.	3–4/12	19.3–22.0	101	28	1302–1443	3.80–4.71	13	++++	++++	2.4–4.5
S. L.	6–5/12	32.4–33.6	118	20	1260–1521	3.07–4.57	11	++++	++++	9.0–9.2
D. W.	3–4/12	16.2–16.3	95	13	703–817	4.43–5.01	18‡	++++	++	2.7–4.9

* At the beginning and at end of study.
† At onset, had a course of ACTH without apparent effect.
‡ Oral cortisone had been given without effect on two occasions, the last time 12 mos. before this study.

could not be distinguished immunochemically from normal human plasma albumin (2).

Radio-iodinated human γ-globulin was prepared with human γ-globulin, Harvard No. 159-4, isolated from pooled plasma by low temperature-ethanol fractionation (8). The human iron-binding globulin used, Harvard No. 194-2x, was crystallized from Fraction IV-7 (9) and has been immunochemically characterized (10). I^{131} was obtained as carrier-free NaI in sodium bisulfite; the iodide and bisulfite were oxidized with nitrous acid and the solution neutralized with sodium hydroxide (11). A solution of 0.94×10^{-3} M I_2 in 1.33×10^{-3} M KI was added to the I^{131} solution and this mixture was then added to a solution of the protein in 0.2 M $NaHCO_3 - Na_2CO_3$ buffer, pH 9.5 (12). The iodinated iron-binding globulin was calculated to have an average of 3 atoms of iodine per molecule and the iodinated γ-globulin was calculated to have an average of 1.5 atoms of iodine per molecule. The proteins were dialyzed against several daily changes of 0.15 M KI and then against several daily changes of 0.15 M NaCl. Precipitation of protein with trichloracetic acid or with specific antibodies revealed that less than 2 per cent of the total radioactivity of each preparation was free or loosely bound. The iodoproteins could not be distinguished immunochemically from the native analogues used in their preparation (2).

Preparation of specimens for analysis: Urine collection periods varied from several minutes at the beginning of each protein study to several days at the end of each study. Two or 3.0-ml. aliquots of urine were placed in screw cap vials for counting; for routine estimation of nonprotein radio-iodide, an additional aliquot of urine was mixed with an equal volume of 20 per cent trichloracetic acid, allowed to stand at 1° C for 6 to 24 hours, centrifuged, and aliquots of the clear supernatant placed in vials. Trichloracetic acid precipitation and immunochemical precipitation gave almost identical results for nonprotein radio-iodide. The difference between the total activity of the urine sample and that of the nonprotein radio-iodide in that sample was taken as the activity due to intact radio-iodinated plasma protein.

After venipuncture, blood was allowed to clot and the serum separated for counting. Serum rather than plasma was used, since the same specimen that was assayed for radioactivity could then be used for immunochemical estimations.

In this report, serum and plasma concentrations of the plasma proteins studied have been considered equivalent. Periodically, aliquots of blood were mixed with sodium oxalate for estimation of hematocrit.

Edema fluid was obtained near the termination of each plasma protein disappearance study in the three patients of Group III. A No. 19 spinal needle, 1.5 inches long and with stylet in place, was inserted into the subcutaneous tissues of the leg in a dependent position; upon removing the stylet, fluid without any traces of red cells flowed freely directly into a centrifuge tube. Unfortunately, serial sampling of edema and ascitic fluids was not done.

Assay of radioactivity: A well-type, sodium iodide crystal scintillation counter was employed which gave 9.4×10^5 counts per minute per microcurie of I^{131}. All

specimens in 2.0 or 3.0-ml. aliquots were placed in 15 by 45 mm. screw cap vials for counting. Radioactive standards were prepared for each iodinated plasma protein studied for each individual patient. Using the same syringe and needle that had been used for the injection and an aliquot of the same solution of radioactive protein used in that instance, a dilution of the injected material was prepared and aliquots of 2.0 and 3.0 ml. were placed in vials as above; these served as standards for the appropriate volume of unknown, thus avoiding problems of decay and geometry.

Immunochemical methods: Rabbit antisera *vs.* crystallized human serum albumin, human γ-globulin and crystallized human iron-binding globulin were prepared as previously described (2, 10). The spectrophotometric method was used for analysis of specific precipitates (7). All specific protein values in this report are based on immunochemical estimations.

Determination of plasma volume: The plasma volume in each instance was taken as the total activity injected less the total amount of activity found in the urine in 12 to 16 minutes divided by the activity in the serum obtained from venous blood taken in 12 to 16 minutes. The total amount of activity in the urine at the end of this period was always less than 0.5 per cent of the amount injected, even in the patients of Group III, and although the plasma volumes were not corrected for catabolism or extravascular diffusion of iodoprotein during this period of time, these corrections were of similar order of magnitude.

Definitions of terms: For the sake of clarity, the following definitions are given:

The *body pool* or *total exchangeable pool* of a given plasma protein is the total amount of that protein in the body which is available for exchange with additional or newly synthesized molecules of that specific protein.

The *turnover half-time* of a plasma protein is the amount of time required for half of the body pool of that protein to be lost to the body through all channels of loss. The *turnover half-time due to catabolism* (or urinary loss) is the time required for half of the body pool of the protein in question to be lost through catabolism (or urinary loss). The *fractional rate of catabolism* (or *urinary loss*) is that fraction of the body pool of a given protein that is catabolized (or excreted) per unit time. *Turnover-time* or *half-life* are expressions that are frequently used instead of turnover half-time; the fractional rates of catabolism and urinary loss are turnover rates.

The *rate of synthesis, catabolism,* or *urinary* loss is the amount of protein that is synthesized, catabolized, or lost in the urine per unit time, respectively.

The *volume of distribution* of a protein is taken as the volume that a protein would occupy if the total exchangeable pool of the protein had a uniform concentration equal to the plasma concentration of that protein. It is to be *definitely* understood that this term has *no* ana-

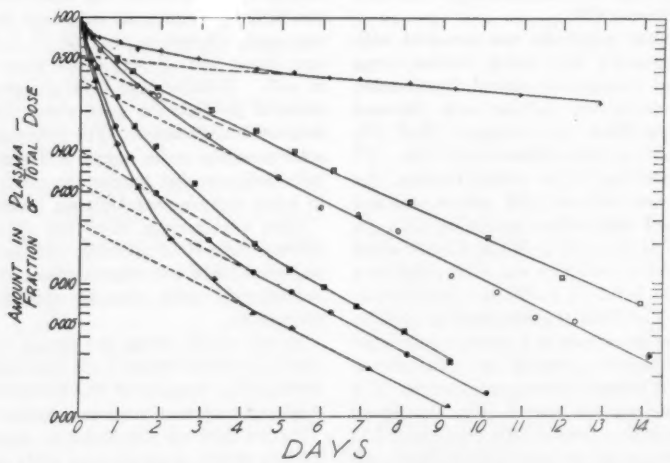

FIG. 1. THE DISAPPEARANCE OF IODINATED PLASMA ALBUMIN FROM THE CIRCULATION AFTER INTRAVENOUS INJECTION

In this figure and in all subsequent figures, the following symbols apply:

+ R. S. B.
□ A. B.
○ E. T.
■ K. S.
● S. L.
▲ D. W.

TABLE II

Rates of urinary loss, catabolism, and synthesis of albumin, γ-globulin and iron-binding globulin

Patient	Serum concentration (*gm. %*)	Plasma volume (*ml.*)	Renal clearance of specific protein (*ml./min.*)	Estimated cumulative urinary losses		Rates		
				Iodinated protein*	Nonprotein iodide†	Urinary loss‡ (*gm./day*)	Catabolism§ (*gm./day*)	Synthesis‖ (*gm./day*)
A: Albumin								
I—R. S. B.	4.33	1405	0.015	0.15	0.85	0.95	5.38	6.33
II A. B.	0.86	1156	0.166	0.27	0.73	2.06	5.57	7.63
II E. T.	0.77	1283	0.472	0.75	0.25	5.22	1.74	6.96
III K. S.	0.58	749	0.236	0.32	0.68	1.96	4.17	6.13
III S. L.	0.40	1001	0.794	0.61	0.39	4.61	2.95	7.56
III D. W.	0.61	701	0.246	0.27	0.73	2.15	5.81	7.96
B: γ-Globulin								
I—R. S. B.	0.611	1236	0.004	0.07	0.93	0.035	0.465	0.500
II A. B.	0.390	1152	0.078	0.09	0.91	0.112	1.13	1.24
II E. T.	0.219	1388	0.148	0.69	0.31	0.466	0.209	0.675
III K. S.	0.186	856	0.076	0.23	0.77	0.110	0.368	0.478
III S. L.	0.193	1008	0.134	0.52	0.48	0.372	0.343	0.715
III D. W.	0.189	733	0.013	0.07	0.93	0.035	0.465	0.500
C: Iron-binding globulin								
II A. B.	0.0280	1388	0.117	0.20	0.80	0.0472	0.189	0.236
II E. T.	0.0285	1374	0.372	0.57	0.43	0.153	0.115	0.268
III—S. L.	0.0145	1191	0.360	0.48	0.52	0.0752	0.0816	0.157

* As fraction of total dose: A^*/A_s^* in Equation (*1*).
† As fraction of total dose: $1-A^*/A_s^*$.
‡ A_s in Equation (*1*).
§ A_s in Equation (*2*).
‖ A_s in Equation (*1*).

tomical implications and does *not* indicate the extent of distribution of the protein in the body; knowing the plasma concentration of a protein, the volume of distribution indicates, among other things, the size of the body pool.

RESULTS

I. *Studies with Radio-iodinated Human Plasma Albumin*

A. *The disappearance of iodinated plasma albumin from the circulation:* The disappearance of radio-iodinated albumin from the plasma of the six children is shown in Figure 1; it should be noted that the ordinates are logarithmic. Simple graphic analysis of these curves resulted in the biological half-life values for total body albumin that are indicated in Table IIIA. The half-life of albumin in the patient of Group I would appear to be within normal limits (13, 14); the half-life of albumin in the patients of Groups II and III would appear to be greatly diminished.

Upon extrapolation of the exponential portions of the disappearance curves to zero time, at first glance it would appear that the children with anasarca and ascites had abnormally large volumes of distribution. Even correcting the curves of Figure 1 by the method of Berson, Yalow, Schreiber, and Post (14), the apparent volume of distribution in the latter patients remained 7 to 10 times the plasma volume. Calculation of the amount of albumin [2] actually present in edema

[2] For example, patient S. L.: Weight: 32 Kg. Height: 118 cm. Estimated edema-free weight: 20 Kg.

Hence, excess fluid = 32 Kg. − 20 Kg. = 12 Kg. and of this, 3 Kg. was ascites. If the normal interstitial fluid space in humans is assumed to be roughly 20 per cent of the body weight, the normal amount of interstitial fluid in this patient in the edema-free state would be 20 per cent of 20 Kg. or 4 Kg. The total interstitial fluid in the edematous state less the amount of ascitic fluid would then be (12 Kg. − 3 Kg.) + 4 Kg. = 13 Kg.; with an albumin concentration of 0.01 gm. per cent, this fluid would account for about 1.3 gm. of albumin. Three Kg. of ascitic fluid with an albumin concentration of 0.02 gm. per cent (2) would represent 0.6 gm. albumin. The total extravascular-extracellular albumin would be 1.3 gm. + 0.6 gm. or 1.9 gm. Since the plasma volume was 1001 ml. and the albumin concentration in the plasma was 0.52

and ascitic fluids and plasma, however, indicated that most of the body albumin, as estimated from such volumes of distribution, cannot be accounted for in these spaces. This apparent discrepancy cannot easily be explained on the basis of albumin being bound to such structures as collagenous fibers, nor can the discrepancy satisfactorily be attributed to a "pool" of intracellular albumin (10). At no time in any of the patients was the plasma nonprotein radio-iodide more than 10 per cent of the total activity of the plasma sample, and hence the problem is not a result of counting nonprotein radio-iodide with radio-iodinated albumin.

gm. per cent, the total vascular albumin was 5.2 gm. Total body albumin in this patient, therefore, was 1.9 gm. + 5.2 gm. or 7.1 gm. The volume of distribution of albumin as estimated from the plasma disappearance curve after an attempt at correction (14) was 7100 ml.; the body pool of albumin on this basis should have been

$$7100 \text{ ml.} \times \frac{0.52 \text{ gm. albumin}}{100 \text{ ml.}} = 36.9 \text{ gm.}$$

Thus, if the latter were correct, 36.9 gm. − 7.1 gm. or 29.8 gm. of albumin cannot be accounted for.

Direct examination of the edema fluids obtained from the children in Group III, however, revealed that in the period of exponential decline of iodoalbumin in the plasma, the specific activity of albumin in the edema fluid, in counts per minute per gram of albumin, was roughly *30 times* that of the plasma; of the radioactivity in the edema fluid, less than 10 per cent was due to nonprotein radio-iodide. Multiplying the specific activity of the albumin in edema fluid by the amount of endogenous albumin present in the extravascular fluids[2] indicated that the total amount of radioactivity present extravascularly at the time of sampling the edema fluid was roughly 9 to 10 times that in the plasma.

Thus, it would appear that the large volume of distribution obtained in each of the patients of Group III represented the final volume of distribution of the iodoalbumin administered and not that of the body pool of endogenous albumin. Actually, this situation should be expected in a three- or more compartment open system such as

TABLE III

Turnover of total body albumin, γ-globulin and iron-binding globulin

Patients	Specific protein in vascular compartment* (gm.)	Estimated total pool of specific protein† = T_A (gm.)	From plasma disappearance (days)	Approximate half-time of turnover: From Equation (4) $t_{\frac{1}{2}}$ (days)	Urinary loss‡ $t_{\frac{1}{2}}$ (days)	Catabolism‡ $t_{\frac{1}{2}}$ (days)	Fractional rates of turnover: Urinary loss (per day)	Catabolism (per day)
A: Albumin								
I—R. S. B.	60.8	135.2	12.0	14.7	98.8	17.5	0.007	0.040
II A. B.	9.94	22.1	2.3	2.0	7.5	2.8	0.093	0.252
II E. T.	9.88	22.0	2.0	2.2	2.9	8.7	0.237	0.079
III K. S.	4.34	9.6	1.8	1.1	3.3	1.7	0.204	0.434
III S. L.	4.00	8.9	2.1	0.8	1.4	2.1	0.518	0.331
III D. W.	4.28	9.5	2.0	0.8	3.1	1.1	0.226	0.612
B: γ-Globulin								
I—R. S. B.	7.55	16.8	22.0	23.3	33.3	25.1	0.002	0.028
II A. B.	4.49	9.98	3.8	5.5	61.8	6.1	0.011	0.113
II E. T.	3.04	6.76	3.7	6.9	10.1	22.4	0.069	0.031
III K. S.	1.59	3.54	3.4	5.1	22.3	6.6	0.031	0.104
III S. L.	1.95	4.32	4.1	4.1	8.0	8.7	0.086	0.079
III D. W.	1.39	3.08	3.3	4.5	61.1	4.6	0.011	0.151
C: Iron-binding globulin								
II A. B.	0.389	0.864	1.7	2.5	12.8	3.2	0.054	0.219
II E. T.	0.392	0.871	1.9	2.2	3.9	5.3	0.176	0.132
III—S. L.	0.173	0.384	2.5	1.7	3.6	3.3	0.196	0.213

* Serum concentration × plasma volume.
† See text: T_A = serum concentration × plasma volume ÷ 0.45.
‡ Equation (3).

was the case in these children; mathematical analysis of such a system reveals that at no time will uniform specific activities exist throughout the system (15). Under these circumstances, plasma disappearance curves cannot be used directly for estimation of the half-life or the volume of distribution of a body pool of endogenous protein (15).

These findings, however, do *not* militate against the assumption that radio-iodinated albumin is a suitable tracer for endogenous albumin. While the body pool of albumin in these children was presumed to be in a steady state in that the pool contained constant amounts of albumin with a given anatomical distribution in the body, it is almost unnecessary to state that the pool was in a continuous state of flux; albumin was lost from the pool by catabolism or excretion and was constantly replaced by newly synthesized albumin molecules. As will be shown later, intravenously administered iodoalbumin behaves as does newly synthesized albumin.

B. *Calculation of rates of catabolism, urinary loss and synthesis of albumin:* It should be *carefully* noted that, in the calculations which follow, several assumptions were made: 1) Radio-iodinated albumin is a suitable tracer for native albumin under the conditions of these studies, that in every way it behaves as does native albumin and is handled by the body as is native albumin; 2) nonprotein radio-iodide appearing in the urine is derived from catabolized albumin, *i.e.*, the iodoalbumin is not simply deiodinated, but the molecule is catabolized in a manner analogous to the catabolism of native albumin; 3) the patient was in a steady state with respect to total circulating albumin and total body albumin.

That the patients could be considered to be in a steady state with respect to the amount of albumin in the body and in the circulation during the time period of this study was indicated by the following: 1) The maximum variation in serum albumin concentration as determined immunochemically throughout the study was ± 10 per cent; 2) the plasma volumes at the beginning of the study as estimated with iodoalbumin agreed reasonably well with those determined at the end of the study period using iodo-γ-globulin (Table II); 3) the maximum weight gain over this period was 1.2 kilograms in the case of S. L., 0.1 and 2.7 kilograms for D. W. and K. S. The weight gain in these children was presumably due to accumulation of edema and ascitic fluids which have an exceedingly low protein concentration (2). The maximum weight gain in the remaining children was 0.1 to 1.1 kilograms.

The cumulative urinary excretion of radio-iodinated albumin and of nonprotein radio-iodide in these patients is plotted in Figures 2 and 3. Correcting for the loss of iodoalbumin from the system because of blood sampling, 2 per cent to 3 per cent of the total dose, the estimated fraction of the total dose represented by urinary loss of iodoalbumin is charted in Table IIA.

If one assumed that albumin, when synthesized, enters the vascular [8] compartment before being distributed *generally* to the interstitial fluid, then it can be shown that the total albumin synthesized per unit time, A_s, can be obtained from the following expression:

$$\frac{A_s}{A_e} = \frac{A_s^*}{A^*} \quad \text{or} \quad A_s = \frac{A_e}{A^*/A_s^*} = \frac{A_e}{A_f^*} \qquad (1)$$

where A_e is the amount of endogenous albumin lost in the urine per unit time, A_s^* is the total dose of radio-iodinated albumin, A^* is the amount of radio-iodinated albumin lost in the urine after an infinite time period, and A_f^* is the fraction of total dose of labelled albumin lost in the urine as labelled albumin.

The amount of native albumin appearing in the urine, A_e, was estimated immunochemically and the results appear in Table IIA. The amount of albumin synthesized per day was then calculated from Equation 1 and charted in Table IIA. Assuming that the loss of albumin from the body is due either to catabolism or urinary excretion, the amount of albumin catabolized per day, A_c, was calculated:

$$A_c = A_s - A_e \qquad (2)$$

It will be noted that the amount of albumin synthesized per day as calculated for these chil-

[8] The bulk of evidence appears to indicate the liver as the source of albumin; it would then appear that this assumption is not unreasonable. Even assuming for a moment that the protein in question was synthesized and passed into the circulation *via* the lymphatics, the protein is transported to the circulation before passing into the interstitial fluids in general. The latter would probably be more to the point for γ-globulin, since this protein appears to be synthesized in the plasma cells of lymph nodes (16, 17) in addition to other sites (18).

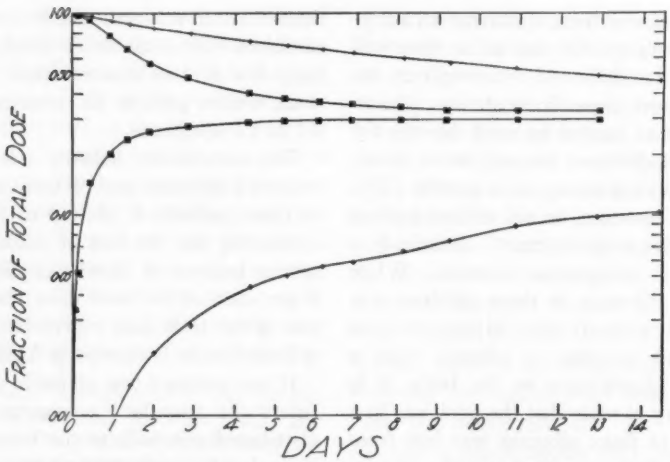

FIG. 2. THE CUMULATIVE URINARY EXCRETION OF RADIO-IODINATED ALBUMIN (CURVES ASCENDING FROM TIME ZERO) AND 1.00 MINUS THE CUMULATIVE URINARY EXCRETION OF NONPROTEIN RADIO-IODIDE OF A PATIENT WITH FULL-BLOWN NEPHROTIC SYNDROME COMPARED WITH THAT OF THE PATIENT WHO RECOVERED FROM THIS DISEASE (ORDINATES ARE LOGARITHMIC)

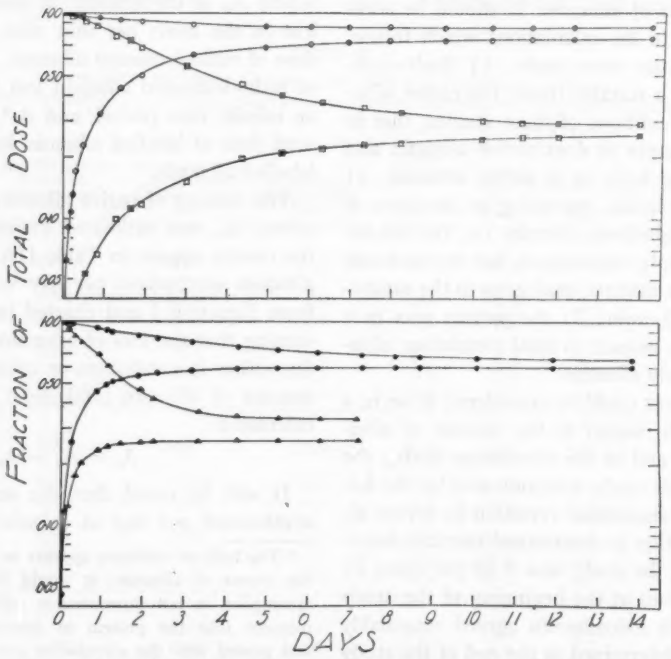

FIG. 3. THE CUMULATIVE URINARY EXCRETION OF RADIO-IODINATED ALBUMIN (CURVES ASCENDING FROM TIME ZERO) AND 1.00 MINUS THE CUMULATIVE URINARY EXCRETION OF NONPROTEIN RADIO-IODIDE OF TWO PATIENTS OF GROUP II (UPPER CHART) COMPARED WITH THAT OF TWO PATIENTS OF GROUP III (LOWER CHART) (ORDINATES ARE LOGARITHMIC)

dren is in the upper limits of the normal range (14).

C. *Crude turnover rates of body albumin due to catabolism and urinary loss:* If the albumin in the vascular compartment represents about 45 per cent of the total body albumin, T_A (13, 14, 19), the half-time of turnover of body albumin due to catabolism, $t_c\frac{1}{2}$, and urinary loss, $t_e\frac{1}{2}$, can be calculated (Table IIIA):

$$t_c\tfrac{1}{2} = \frac{T_A}{A_c/0.693} \quad \text{and} \quad t_e\tfrac{1}{2} = \frac{T_A}{A_e/0.693} \qquad (3)$$

If $t_t\frac{1}{2}$ is the half-life for total body albumin, then:

$$t_t\tfrac{1}{2} = \frac{T_A}{(A_c + A_e)/0.693} = \frac{t_c\frac{1}{2} \cdot t_e\frac{1}{2}}{t_c\frac{1}{2} + t_e\frac{1}{2}} \qquad (4)$$

The fractional rate of catabolism of albumin is the fraction of total body albumin that is catabolized per unit time, or A_c/T_A. Similarly, that fraction of total body albumin lost in the urine per unit time is the fractional rate of urinary loss. These fractional rates are tabulated in Table IIIA.

It would appear that the use of an extravascular albumin of 55 per cent of the total body albumin was not in serious error (+ 5 per cent) in patient R.S.B., as indicated by the extrapolation in Figure 1. This figure, 55 per cent, would represent an upper limit for extravascular albumin for the children of Group II (2, 19). Based on albumin determinations in an earlier series of 20 edematous children with active nephrotic syndrome, it was found, as shown above, that extravascular albumin in patients of Group III constituted about 30 per cent of the total body albumin. Using the figure of 55 per cent, the total body albumin of the children of Group III was probably overestimated; the fractional rates of catabolism and urinary loss for these children, therefore, were actually greater than indicated.

It would appear from the data in Table II that hypoalbuminemia in the patients of Group II could be on a basis of massive albuminuria (patient E.T.) or a markedly increased fractional rate of albumin catabolism (patient A.B.), although elements of increased catabolism and urinary loss were apparent in both patients. The markedly severe hypoalbuminemia seen in the children with ascites and anasarca, however, was apparently the result of combination of both severe albuminuria and greatly increased fractional rate of catabolism of albumin.

It is obvious at once that, without accurate figures for extravascular albumin and total body albumin, these calculations are only approximate, but the significance of even these crude estimations is apparent.

II. *Studies with Radio-iodinated Human γ-Globulin*

A. *The disappearance of iodinated γ-globulin from the circulation after intravenous injection:*

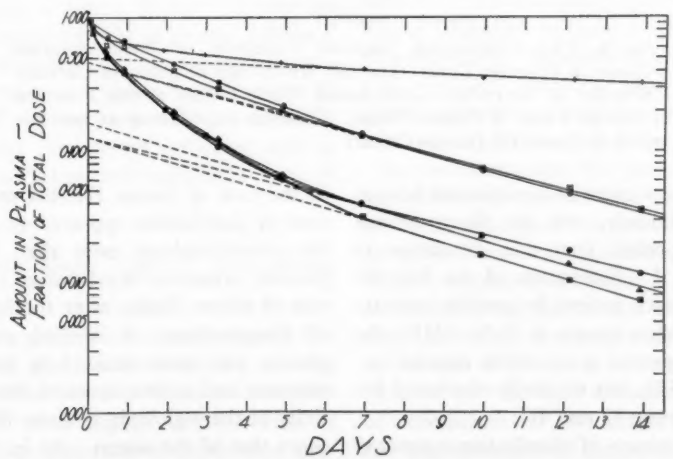

FIG. 4. THE DISAPPEARANCE OF IODINATED γ-GLOBULIN FROM THE CIRCULATION AFTER INTRAVENOUS INJECTION (ORDINATES ARE LOGARITHMIC)

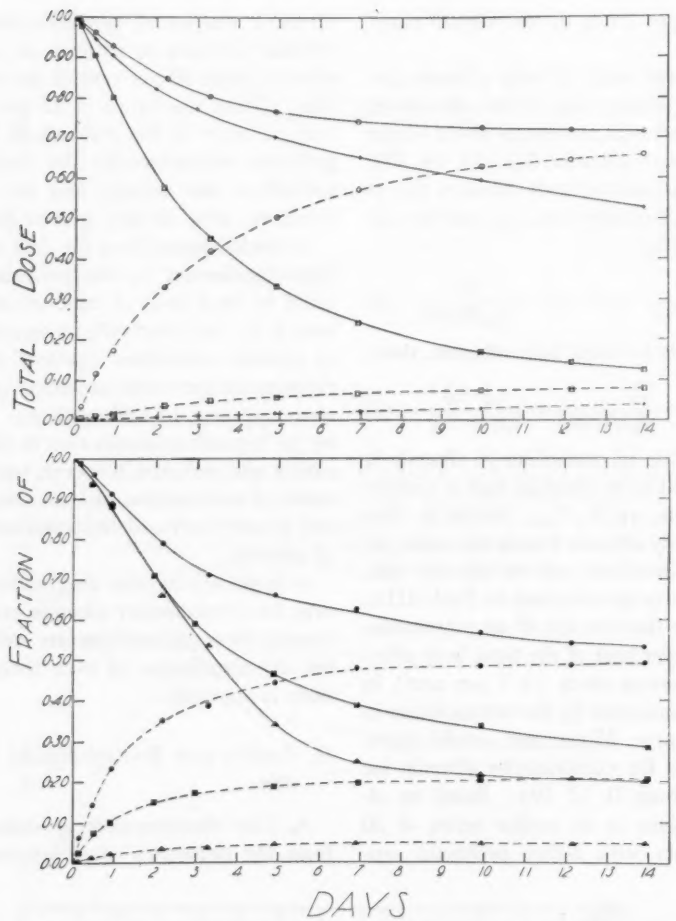

FIG. 5. THE CUMULATIVE URINARY EXCRETION OF RADIO-IODINATED γ-GLOBULIN (DASHED LINES) AND 1.00 MINUS THE CUMULATIVE URINARY EXCRETION OF NONPROTEIN RADIO-IODINE (SOLID LINES) OF THE PATIENTS OF GROUPS I AND II (UPPER CHART) COMPARED WITH THOSE OF THE PATIENTS OF GROUP III (LOWER CHART)

All six children were given radio-iodinated human γ-globulin intravenously, and the disappearance of this labelled protein from the circulation is shown in Figure 4. Estimation of the half-life of the protein in each patient by graphic analysis resulted in the values shown in Table IIIB; the half-life values appeared to be within normal limits for patient R.S.B., but markedly shortened for the children of Group II and III.

The apparent volume of distribution estimated by extrapolation to zero time (Figure 4) appeared within the normal range for Groups I and II, but in the case of Group III, the extravascular volume of distribution appeared to be 5 to 6 times the plasma volume even after "correction" for possible variations in catabolism (14). Examination of edema fluids, after the logarithmic phase of disappearance of labelled protein from the plasma had been attained in the children with anasarca and ascites, revealed that the specific activity of the γ-globulin in these fluids was some 8 times that of the serum. As in the case of iodinated albumin, this factor apparently would: 1) Increase the half-life of iodinated γ-globulin in the

circulation through a feedback; 2) indicate a much larger volume of distribution for iodinated γ-globulin than is actually the situation for native γ-globulin in these patients.

The biological half-life of this preparation of radio-iodinated γ-globulin in three normal children was 17 to 22 days.

B. *Rates of catabolism, urinary loss and synthesis of γ-globulin:* The same assumptions which were necessary for the albumin calculations were applied to the study of γ-globulin. The cumulative urinary excretion of radio-iodinated γ-globulin and of nonprotein radio-iodide for these children are shown in Figure 5; it is to be noted that both coordinates are linear simply to indicate how these losses appear on this type plot instead of a semilogarithmic plot.

Calculations based on the same considerations of urinary losses as detailed for radio-iodinated albumin resulted in the values for catabolized and synthesized γ-globulin indicated in Table IIB. It would appear from the data that in the nephrotic syndrome, synthesis of γ-globulin may be normal or somewhat accelerated.

C. *Crude turnover rates of total γ-globulin due to catabolism and urinary loss:* Using the same arguments as for iodinated albumin, the half-times of turnover of total body γ-globulin attributable to catabolism and urinary loss were estimated, as well as the corresponding fractional rates of turnover (Table IIIB). It would appear that in one patient of Group II and two patients of Group III, catabolism was of greater influence in keeping the patient in a hypogammaglobulinemic state than was urinary loss; in one patient of Group II, E. T., the urinary loss of γ-globulin appeared to exert the greater influence, and in S. L., both urinary loss and catabolism were of equal influence.

III. *Studies with Radio-iodinated Iron-binding Globulin*

Three patients were given radio-iodinated human iron-binding globulin intravenously and the plasma disappearance studied; these patients were the two children of Group II and one child of Group III, S. L., The rates of catabolism, synthesis and urinary loss of iron-binding globulin and the turnover rates of this protein due to catabolism and urinary loss were calculated and are charted in Tables IIC and IIIC. The biological half-life for this radio-iodinated iron-binding globulin preparation in a normal child was 12 days.

DISCUSSION

It is apparent that in children with the nephrotic syndrome, hypoproteinemia is accompanied by a reduced plasma protein pool. For adequate interpretation of the data, it is essential to know whether a reduction in the plasma protein pool occurred as a result of an increased fractional rate of catabolism or whether body requirements demanded the catabolism of a certain minimum amount of plasma protein. In the latter instance, a reduced plasma protein pool resulting from other causes, such as urinary loss, would give rise to an increased fractional rate of catabolism.

Severe reduction in the total body pool of plasma protein in dogs by plasmapheresis (20, 21) and in mice by chronic bleeding (20), did not increase the fractional rate of catabolism of homologous plasma albumin. Similarly, the extreme or complete reduction in the body pool of a single plasma protein due to failure in synthesis, such as in congenital agammaglobulinemia (22) or congenital afibrinogenemia (23), does not result in any increase in the fractional rate of catabolism of that protein. In addition, it will be noted that in patient E. T., despite a marked reduction in total body albumin and γ-globulin, the fractional rate of catabolism of these proteins was not markedly increased. Available evidence indicates that the increased fractional rate of catabolism of plasma protein reported here was not secondary to a reduced plasma protein pool.

In the steady state, the rate of albumin synthesis must equal the rate of loss of albumin from the total body pool. It would appear from the data presented that hypoalbuminemia in children with the nephrotic syndrome is due to an increase in the fractional rate of loss of albumin from the body pool of greater magnitude than any increase in the rate of synthesis, and that this increase in turnover of total body albumin may be the result of a combination of two factors: 1) An increase in the fractional rate of catabolism of albumin; and 2) albuminuria. Moderate hypoalbuminemia, such as that seen in the two children without mani-

fest ascites but with minimal edema, apparently can be due either to: 1) A relatively severe albuminuria with a moderate increase in the fractional rate of albumin catabolism; or 2) a greatly increased fractional rate of catabolism of albumin in association with but a relatively moderate degree of albuminuria. In children with ascites and anasarca, however, the fractional rate of albumin catabolism and the renal loss of albumin are *both* greatly increased. In the child who had recovered from the nephrotic syndrome, but who still had a mild proteinuria, the rate of albumin catabolism was apparently within normal limits.

The hypogammaglobulinemia found in this disease can similarly be explained by an increase in γ-globulin turnover due to an increase in the fractional rate of catabolism of γ-globulin and by losses of γ-globulin from the circulation through the renal glomerulus. Thus, almost all of the children of Groups II and III showed increased fractional rates of catabolism; in one child of Group II (E. T.), the increase in turnover of body γ-globulin could be attributed to renal loss rather than to any increase in catabolism.

The deficiency in circulating iron-binding globulin seen in children with the nephrotic syndrome (24) apparently is attributable to the same factors found for γ-globulin and albumin; *viz.*, renal loss and an increase in the fractional rate of catabolism of this protein.

The rates of synthesis of albumin and iron-binding globulin in these children, in grams per unit time, were found to be at the upper limits of the normal range. While the rates of synthesis of γ-globulin also were generally found to be within normal limits, in one child the rate of synthesis was definitely above normal. Certainly, the rates of synthesis determined are somewhat lower than the 2.5 to 5-fold increase in plasma protein synthesis suggested by others (1, 25). This is in accord with the finding that in animals kept on diets normal in protein content and who have been severely depleted of plasma protein by plasmapheresis or by chronic bleeding, the rate of synthesis of albumin was the same as that in normal controls (20).

Using S^{35}-methionine (25) and N^{15}-glycine (26), other investigators have found an increased incorporation of these labels into new plasma protein in the nephrotic syndrome; this has been interpreted as indicating a greatly accelerated rate of plasma protein synthesis in this disease. It has been indicated in this report, however, that the turnover rates of the plasma proteins studied may be greatly increased during the nephrotic state and that the concentrations of these proteins in the plasma were much lower than normal; under these circumstances, and especially in the presence of a reduced amino acid pool (27), the rate of label incorporation as measured by the rapid increase in specific activity need not necessarily reflect an increased rate of synthesis of these proteins in terms of grams per day.

In a study of protein and carbohydrate metabolism in nephrotoxic nephritis induced in rats, Drabkin and Marsh (28) have just reported a similar increase in incorporation of C^{14} in tissue proteins as well. They concluded that an increased turnover of body protein occurred in the nephrotic rat. They also concluded that the metabolic adjustments found in the rat were related to proteinuria in that the metabolic pathways were channeled into increased protein synthesis in an attempt to adjust to renal excretion of protein (29). In several of the children reported here, the fractional rate of catabolism, however, was of greater influence on the turnover of plasma protein than was the fractional rate of renal excretion, and protein synthesis was not greatly increased in any.

During the course of this report, the reader has been subjected to a seemingly inordinate series of assumptions which may be true only within certain limits. For simplification, in the presentation of the results of this study, it was assumed that the radio-iodinated plasma proteins used in this investigation were satisfactory for studying the catabolism of the native proteins. This was done with full realization that this assumption may not have been strictly true. In children with congenital agammaglobulinemia, for example, it has been found that the rate of catabolism of radio-iodinated γ-globulin is considerably faster than that of unlabelled γ-globulin (22). To test this possibility, Table IV was constructed assuming that the catabolized fraction of the native albumin had been overestimated by a factor of 2; it can be seen that the general conclusions reached need not be radically changed. If, by unusual circumstance, the iodinated albumin had been catabolized

TABLE IV

Rate of turnover of endogenous albumin if the fraction of albumin catabolized had been overestimated by a factor of 2

Patient	$\frac{A^*}{A_s^*}+\frac{1}{2}\left(1-\frac{A^*}{A_s^*}\right)$ (From Table IIA)	$\frac{1}{2}\left(1-\frac{A^*}{A_s^*}\right)$ (From Table IIA)	Rates: Synthesis (gm./day)	Rates: Urinary loss (gm./day)	Rates: Catabolism (gm./day)	Approximate half-time of turnover: $t_{\frac{1}{2}}$ (days)	Approximate half-time of turnover: $t_{e\frac{1}{2}}$ (days)	Approximate half-time of turnover: $t_{s\frac{1}{2}}$ (days)	Fractional rates of turnover: Urinary loss (per day)	Fractional rates of turnover: Catabolism (per day)
I—R. S. B.	0.57	0.43	1.67	0.95	0.72	56.2	98.8	13.0	0.007	0.005
II A. B.	0.63	0.37	3.27	2.06	1.21	4.7	7.5	12.8	0.093	0.055
II E. T.	0.87	0.13	6.00	5.22	0.78	2.5	2.9	19.6	0.237	0.035
III K. S.	0.66	0.34	2.97	1.96	1.01	2.2	3.3	6.7	0.204	0.105
III S. L.	0.80	0.20	5.76	4.61	1.15	1.1	1.4	5.4	0.518	0.129
III D. W.	0.63	0.37	3.41	2.15	1.26	1.9	3.1	5.1	0.226	0.133

more slowly than the native albumin, the fractional catabolic rate for the iodinated albumin would have had to fall to less than ⅓th of its normal rate to alter the conclusions.

SUMMARY

Six children in various phases of the nephrotic syndrome were given tracer doses of radio-iodinated human plasma albumin and radio-iodinated human γ-globulin intravenously in sequence. Three of these children were then given intravenous injections of radio-iodinated human iron-binding globulin. The disappearance of specific radio-iodinated plasma protein from the circulation and its cumulative appearance in the urine were studied; the urinary excretion of nonprotein radio-iodide was also investigated. Unlabelled albumin, γ-globulin, and iron-binding globulin in serum and urine, and in several instances in edema fluid, were estimated immunochemically and the renal clearances of these proteins determined.

Calculations indicated that the half-time of turnover for native plasma albumin could not be estimated from the plasma disappearance curves of the radio-iodinated analogue in the edematous patient; in the phase of exponential disappearance from the circulation, the specific activity of the given protein in the extravascular fluid was found to be 8 to 30 times that in the plasma. The large apparent volume of distribution obtained by extrapolation of the exponential portion of these curves is, at least in part, a reflection of this difference in specific activities and while the extrapolation indicates the volume of distribution of the radio-iodinated plasma protein during this phase, it does not measure the volume of distribution of the native protein in the steady state.

The deficiencies of albumin, γ-globulin and iron-binding globulin seen in the plasma of children with the nephrotic syndrome are due to an increased fractional rate of catabolism of these proteins in association with renal losses. Moderate hypoalbuminemia without apparent ascites but with minimal edema may be associated with *either* a greatly increased fractional rate of catabolism of albumin *or* a severe degree of albuminuria; severe hypoalbuminemia with ascites and anasarca is the result of *both* greatly increased catabolism and severe albuminuria occurring simultaneously.

The rates of synthesis of albumin, γ-globulin and iron-binding globulin in these children were at the upper limits of the normal range except that in one instance, an increased rate of γ-globulin synthesis was found.

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APPENDIX

The system considered in these children for the calculation of the rate of synthesis of endogenous plasma protein is a simple one in which the endogenous or carrier protein enters and leaves the system at a rate, A_s, and is excreted from the system at a rate, A_e. If R^* is the radioactivity in the system at any given time, R^* is a function of time:

$$R^* = \Phi(t)$$

and has a rate of change:

$$\frac{dR^*}{dt} = -\frac{d\Phi(t)}{dt}$$

Assuming the tracer behaves like the carrier:

$$\frac{A_e}{A_s}\frac{dR^*}{dt} = -\frac{A_e}{A_s}\frac{d\Phi(t)}{dt}$$

Solving for the amount, A*, of tracer excreted from time 0 to T:

$$\int_0^T \frac{A_e}{A_s}\frac{dR^*}{dt} = \int_0^T -\frac{A_e}{A_s}\frac{d\Phi(t)}{dt}$$

$$A^* = -\frac{A_e}{A_s}\frac{d\Phi(t)}{dt}\Big]_0^T$$

If at $t = 0$, $\Phi(t) = A_s^*$, where A_s^* is the total amount of radioactivity injected and at $t = \infty$, $\Phi(t) = 0$, then

$$A^* = \frac{A_e}{A_s}A_s^*$$

or

$$A_s = \frac{A_e}{A^*/A_s^*}$$

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RESPONSE TO TRIIODOTHYRONINE AS INDEX OF PERSISTENCE OF DISEASE IN THE THYROID REMNANT OF PATIENTS IN REMISSION FROM HYPERTHYROIDISM[1]

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The present study was designed to determine the duration of activity of disease in the thyroid remnant of patients in sustained remission following treatment for hyperthyroidism. Failure of I-131 uptake to be decreased significantly by triiodothyronine administration was used as the criterion of persistence of disease. The recent observations that thyroid (1, 2) and triiodothyronine (3–5) do not decrease I-131 uptake when given to patients with overt disease indicate that the lack of response to these agents is fairly specific. Subjects without thyroid disease (1, 6–8) or patients with thyroid disorders other than hyperthyroidism show a sharp reduction in uptake; the one exception is found in nontoxic nodular goiter (4, 5) in which about 1 in 4 do not respond. The glands of such patients contain "hot," *i.e.*, highly active, nodules.

Rienhoff (9) and Roussy, Huguenin, and Welti (10) investigated the duration of thyrotoxicosis in the gland fragment remaining after surgery, using hyperplasia as a criterion. They studied the glands of six euthyroid patients coming to biopsy up to six years after operation, and found in all persistence of hyperplasia. Using the response to triiodothyronine as the criterion, the present investigation reveals that activity of disease within the gland remnant may persist for more than 20 years after remission was induced by one of the modalities of treatment; or may subside within a few months of the onset of remission. Abnormal responses appear to predominate in the first five years after I-131 therapy whereas the number of normal responses in the same time period following surgery equals or is greater than the abnormal ones. Normal responses predominate after the five-year interval in both groups.

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METHODS

All patients tested had had documented toxic diffuse or toxic nodular goiter and had been in remission for various lengths of time before the study was made. The duration of euthyroidism prior to testing with triiodothyronine is recorded in Table I.

Most of the patients were originally seen, treated, and followed subsequently, in the Thyroid Clinic of the Presbyterian Hospital in New York. The usual modalities of treatment are represented in the series. Just prior to testing with triiodothyronine, all patients were examined by the writer and were sent to the laboratory for determinations of basal metabolic rate, fasting serum cholesterol and 24-hour I-131 uptake by standard methods. The serum precipitable iodine level was also measured by the original Barker method (11) when there was disagreement among the other tests. Only those patients are included in the series who seemed unquestionably euthyroid and who had been so, as best as could be determined, for the interval stated in the table.

Following the above appraisal, the patients were tested with triiodothyronine according to the method described elsewhere (4). After determination of the 24-hour I-131 uptake, each patient was given l-sodium triiodothyronine by mouth, 75 μgm. daily for 8 days. On the eighth day, a count was made over the thyroid, to account for radioactivity remaining from the preceding test. A second test dose of I-131 was then administered and uptake measured 24 hours later. Test doses of 15 μc I-131 each were employed. A bismuth cathode Geiger tube was used for counting over the thyroid region with the counter placed 15 cm. from the skin and able to see an area about 10 × 10 cm. Counting time was about 10 minutes (2000 counts) to provide a counting accuracy of about 3 per cent with the background count subtracted.

Throughout, a response was termed "normal" when the final I-131 uptake value after triiodothyronine administration was less than 20 per cent, or was not more than 60 per cent of the initial value when the initial uptake was already low. There were virtually no values in this group with a final value as much as 50 per cent of the initial value. The response was termed "abnormal" if the final value was 30 per cent or more, or was 60 per cent or more of the initial value when the initial uptake was low.

TABLE I

Table showing duration of euthyroidism, type of treatment, and distribution of responses to triiodothyronine in patients in remission from hyperthyroidism following treatment

Therapy	Patient category	Duration of euthyroidism (*Years*)													
		<1	1	2	3	4	5	6	7	8	9	10–14	15–19	20–	Total
I-131	Normal * (No. of patients)	1	2	1	5	1	1	4	2	1					18
	Abnormal * (No. of patients)	11	9	10	4	8	4	0	1	1					48
Operation	Normal (No. of patients)	1	6	1	1	1	1	1	1	1	0	1	2	0	17
	Abnormal (No. of patients)	0	2	2	0	1	0	0	0	0	1	2	0	2	10
Antithyroid	Normal (No. of patients)	0	0	1	0	0	0	1	2	0	1	0	0	0	5
	Abnormal (No. of patients)	1	0	1	0	0	0	0	0	1	0	1	0	0	4
Miscellaneous	Normal (No. of patients)	0	0	0	0	0	1†	0	0	0	0	0	0	1‡	2
	Abnormal (No. of patients)	0	1‡	0	0	0	0	0	0	0	0	0	0	0	1
														Total	105

* For definition, see text under "methods."
† Remission following radiotherapy.
‡ Spontaneous remission.

RESULTS

The results are summarized in Table I. There were 105 patients in four therapeutic categories: 1) I-131 treatment—66 patients; 2) Subtotal thyroidectomy—27 patients; 3) Chronic antithyroid drug therapy (mostly maintained for a year or more before discontinuance)—9 patients; 4) Miscellaneous—1 patient treated by roentgen therapy and 2 patients entering remission spontaneously. A few patients originally with toxic nodular goiter were studied and the results combined with those of the toxic diffuse group because no special trend was observed.

TABLE II

Table showing mean 24-hour I-131 uptake values before and after triiodothyronine administration in patients in remission from hyperthyroidism following treatment by I-131 or operation

Therapy	Duration of remission	Response: Normal — Number of patients	Normal — Average 24 hr. uptake: Initial	Normal — Average 24 hr. uptake: Final	Response: Abnormal — Number of patients	Abnormal — Average 24 hr. uptake: Initial	Abnormal — Average 24 hr. uptake: Final
	Years		%	%		%	%
I-131	0–5	11	28	11	46	34	34
	6–	7	27	10	2	23	18
Operation	0–5	11	25	12	5	38	37
	6–	6	28	8	5	36	38

A. I-131 treatment

There were 66 patients who had been given I-131 from several months to 8 years before testing (Table I). Following triiodothyronine administration, 17 patients showed a normal response (Tables I, II, III). The earliest return to normal was at 4 months after treatment, whereas lack of response, *i.e.*, evidence of activity of disease, was still present almost 9 years after therapy. Prior to 5 years, the ratio of abnormal to normal responses was approximately 5 to 1; subsequently 1 to 3. Statistical analysis of this difference in ratios reveals that $\chi^2 = 10.1$ which is significant at better than the 0.1 per cent level. Also, the mean of the time after I-131 therapy for those with an abnormal response is 3.2 years whereas it is 4.3 years for those with a normal response. This is significant at better than the 1 per cent level.

B. Subtotal thyroidectomy

There were 27 patients in the operative series. Seventeen of these showed a normal response and

TABLE III

Table showing distribution of I-131 uptake values before and following administration of triiodothyronine

	Therapy			
	I-131 Number of uptake values		Surgery Number of uptake values	
% Uptake	Initial	Final	Initial	Final
	Normal response group			
0– 9	0	7	0	8
10–19	2	9	3	9
20–29	10	2	8	0
30–39	4	0	5	0
40–49	2	0	1	0
50–59	0	0	0	0
60–69	0	0	0	0
70–	0	0	0	0
Total	18	18	17	17
	Abnormal response group			
0– 9	0	0	0	0
10–19	4	6	2	1
20–29	11	17	1	2
30–39	15	11	3	2
40–49	5	7	0	2
50–59	3	5	2	2
60–69	2	1	1	1
70–	0	1	0	0
Total	40*	48	9*	10

* Values excluded were determined more than two months prior to the administration of triiodothyronine.

10 an abnormal one. A normal response was noted within 1 year of operation, and abnormal responses were elicited more than 20 years after treatment (Tables I and III). Normal responses tended to preponderate in the first 5 years after operation, the ratio being about 2 normal to 1 abnormal response and 6 to 5 after 5 years (Table II). The difference in the ratio in the first 5 years from that found after I-131 therapy has a significance better than the 0.1 per cent level with $\chi^2 = 11.7$. The number of patients is too small to be sure of significance in the difference in ratios before and after 5 years following surgery.

C. Chronic antithyroid drug therapy

The number of patients in remission following cessation of chronic antithyroid drug therapy is small (Table I). One patient showed no response to triiodothyronine as late as 12 years after remission and on the other hand, a normal response was noted at the earliest time tested, 2 years after remission.

D. Miscellaneous

One patient in this group received external radiation therapy with x-rays. This patient was tested 5 years after remission and exhibited a normal response to triiodothyronine (Table I). There were two patients in spontaneous remission from hyperthyroidism. One showed an abnormal response 1 year after becoming euthyroid, and the other tested 22 years after, had a normal response (Table I).

DISCUSSION

In hyperthyroidism, I-131 uptake by the thyroid is not decreased significantly by the administration of triiodothyronine. This lack of response has been seen otherwise only in nontoxic nodular goiter with "hot" nodules within the gland (4, 5). Lack of response in toxic goiter presumably therefore is indicative of activity of disease. As was noted in the earlier studies, in 10 of 13 patients given thyroxine (12) and 3 of 17 patients given thyroid (1) as well as with this test (4) and documented here, a return to normal responsiveness may occur after sustained remission of hyperthyroidism has been induced by appropriate therapy; this presumably represents subsidence of the disease. Continuance of an abnormal response after remission therefore presumably implies persistence of disease within the gland remnant, despite the euthyroidism. It is unlikely that an abnormally rapid disposal of triiodothyronine, demonstrated in patients with hyperthyroidism and in remission from the disease (13), could account for lack of response of uptake in the present series in view of the daily doses and size of doses which were used and the prolonged duration of treatment, 8 days.

The finding that activity of hyperthyroidism within the thyrotoxic remnant may persist for long periods of time or may subside at varying intervals of the treatment corresponds with the information from earlier histological studies (9, 10). This work revealed by biopsy that hyperplasia had persisted in the thyrotoxic remnant of patients as late as 6 years after remission from successful surgery. The series included only six patients. In a more recent study of seven patients dying of various diseases somewhat later after the onset of remission, histopathological evidence

of persistence of disease was noted as late as 7¾ years, possibly 13 years, after surgery (14). However, the use of hyperplasia as the criterion for persistent activity of disease is open to the criticism that the increased cellular activity could conceivably be a compensatory response to removal of most of the gland.

In the present data, an abnormal response to triiodothyronine is seen for the first 5 years after I-131 in the majority of patients, but thereafter the response of the majority is normal. This implies that there is active disease within the thyrotoxic remnant which was not eliminated by therapy or the establishment of euthyroidism, but which ultimately does subside spontaneously. However, a distinct and highly significant difference from this group is seen when the response to triiodothyronine of the patients treated surgically is examined. Here the majority exhibit a normal response as early as 1 to 2 years after operation as stated above. The difference in time is significant to better than the 0.1 per cent level, although the series is small.

The explanation for the fact that surgical patients seem to revert to normal earlier than I-131 treated patients is not clear. After I-131 therapy, presumably only those cells resistant to radiation effect survive, whereas after surgery the cells which remain possess the same properties as those removed. Subsidence of the disease in the remnant could conceivably be influenced by this factor of selection, the radio-resistant cells being slower to recover. However, it is of interest that an abnormal response to triiodothyronine may persist in antithyroid drug treated patients and following spontaneous as well as induced remission. Another factor of selection lies in the fact that older patients or patients with recurrent hyperthyroidism are usually selected for I-131 therapy and younger ones for surgery. Whether these factors could cause the disparity in results between the 2 groups is subject for speculation.

Besides the abnormality in response to triiodothyronine, there is an abnormal response to thyrotropin in active hyperthyroidism (15), and this may persist after sustained remission has been induced (1). Actually there is a dichotomy of response to thyrotropin: I-131 uptake is unaffected but release of secretion is accelerated as evidenced by an increase in level of serum precipitable iodine (1, 15). The lack of response to thyrotropin of the uptake in patients in remission following treatment for hyperthyroidism has been recently confirmed in another laboratory although no response of the serum precipitable iodine occurred (16). However, a few of the post-treatment patients of the original study and in this later series did develop an increased uptake following thyrotropin injection and an increase in serum precipitable iodine. This normal responsiveness suggests that they might also have exhibited normal responsiveness to triiodothyronine if they had been so tested. This supposition requires documentation.

Uptake of I-131 by the thyroid, also, is not increased by thyrotropin in euthyroid patients with early eye signs of Graves' disease although the serum precipitable iodine level becomes elevated (17). Since the uptake of these patients is well within the normal range, the discrepancy between effects on uptake and release has been taken as added evidence that the derangement is in the thyroid cells or is mediated by unknown mechanisms rather than that a hyperfunctional pituitary because of its increased thyrotropin release has caused the Graves' disease (15). The observation that the activity of disease within the thyroid remnant after treatment for hyperthyroidism limited to the thyroid gland may subside earlier after one modality than after another again suggests that the disorder within the gland is not mediated through the pituitary.

Finally, it appears reasonable to postulate that patients who maintain active disease in the thyroid remnant are the ones who are more apt to develop recurrent hyperthyroidism than those in whom the disease has subsided. It is clear that the triiodothyronine test is positive at the time of recurrence of hyperthyroidism (4). If this assumption be true, the use of triiodothyronine would have prognostic, as well as diagnostic, value. Conversely, it must be remembered in using the test that the patient suspected of recurrent hyperthyroidism may exhibit an abnormal response even though euthyroid.

SUMMARY

1. Duration of activity of disease in the thyroid remnant has been studied in patients in sustained

remission from hyperthyroidism subsequent to treatment by the usual modalities. An abnormal response of I-131 uptake by the thyroid following the administration of triiodothyronine has been taken to indicate such activity.

2. Evidence of active disease within the gland was found at well beyond 20 years after onset of remission and subsidence of disease occurred as early as 4 months after therapy as shown by a return to normal of the I-131 uptake response to triiodothyronine.

3. The majority of patients tested showed evidence of activity of disease within the gland for the first 5 years after I-131 therapy and of subsidence of disease thereafter. By contrast, in the majority of a small group of patients after surgery, the activity of the disease subsided within 1 to 2 years. A few patients treated previously by chronic antithyroid drug therapy, one patient treated by roentgen therapy and two patients in spontaneous remission were also studied.

4. The possibility that the response to triiodothyronine may be abnormal despite euthyroidism in patients previously treated for hyperthyroidism must be taken into account when the triiodothyronine test is used in the diagnosis of recurrent hyperthyroidism.

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THE METABOLIC RESPONSE TO BURNS

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Since Cuthbertson first published his important investigations, the metabolic response to trauma has been the subject of continuous study in many disciplines of medicine (1–8). An impressive body of knowledge has been accumulated with respect to changes in plasma electrolyte concentrations, urinary electrolyte excretion, body composition, and adrenocortical activity.

There is general agreement that injury is accompanied by an increased excretion of nonprotein nitrogenous products, but the importance of this phenomenon, its interpretation in terms of body economy, and its therapeutic implications remain the subject of controversy (4, 5, 7). On the basis of the nitrogen-sulfur and nitrogen-phosphorus ratios in the urine of rats with fractures, Cuthbertson concluded in his earlier studies that the increased excretion of nitrogen after fractures results chiefly from muscle catabolism (1). Howard and Mason, on the other hand, were unable to correlate nitrogen balance with that of potassium or phosphorus in postoperative patients (8). In burned rats, Braasch, Bell, and Levenson noted only transiently increased excretion of potassium and phosphorus while nitrogen excretion remained high for longer periods of time (9). Cope, Nathanson, Rourke, and Wilson (10) and Moore and Ball (7) recorded early negative nitrogen and positive potassium balance in burned patients.

In the present study, the balances of nitrogen, calcium, phosphorus, magnesium, potassium, sodium, and chloride were measured in burned patients for long periods of time. Information was sought about 1) the relative rates of mineral losses, and 2) changes in body composition.

METHODS

The data on five adults, four men and one woman, are included in this report. All were in good health before injury. Metabolic studies were begun immediately after injury. The length of individual studies varied from 60 to 180 days. The early fluid therapy given, the rationale for its use, and the details of local care have been discussed in previous publications (11, 12).

I. *Metabolic ward regimen*

Facilities were available for complete studies on two patients at any one time. The patients were cared for in special cubicles or in the air-conditioned side-rooms of a research ward. Air-conditioning was provided in order to minimize the losses of electrolytes through sweat. One of the authors was responsible for all aspects of treatment. Four nurses who were especially trained in metabolic technics were responsible for nursing care and collection of all specimens. The patients were weighed daily for the first two postburn weeks and twice a week thereafter. A Toledo scale was used with a special attachment for weighing patients on Stryker frames. This scale registers weights to within 114 gm. The reproducibility of weighing a patient, with or without a frame, was repeatedly tested and found to be satisfactory. When possible, allowance for weight change due to a dressing change was made by weighing patients both before and after a dressing change.

II. *Dietary regimen*

All of the food was prepared in and served from a special diet kitchen under the supervision of the research dietitian. Three menus were served in rotation, each menu supplying approximately 2000 calories, 100 gm. of protein, 250 gm. of carbohydrate, 60 gm. of fat, 50 mEq. of sodium, 75 mEq. of potassium, 22 mEq. of magnesium, 45 mEq. of chloride, 1.3 gm. of calcium, and 1.5 gm. of phosphorus. The menus consisted only of foods that can be stored or preserved in the frozen state. A dietary supplement was given in the form of a formula consisting of eggs, dried whole and skim milk, Dextri-Maltose®, and a flavoring agent. The supplement provided an additional 2700 calories, 100 gm. of protein, 473 gm. of carbohydrate, 85 gm. of fat, 130 mEq. of sodium, 120 mEq. of potassium, 135 mEq. of chloride, 22 mEq. of magnesium, 2.4 gm. of calcium, and 2.7 gm. of phosphorus. If a patient ingested all of the menu and the supplement, he received approximately 4700 calories and 200 gm. of protein. The following vitamins were given in capsule form: ascorbic acid—1000 mg.; thiamin hydrochloride—60 mg.; riboflavin—40 mg.; nicotinamide—200 mg.; and calcium pantothenate—40 mg. A serious attempt to achieve constancy of intake was not made

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because of the critical nature of the patients' illness. The patients were encouraged to eat as much of the total diet as possible. The supplement was given between meals during the day and at intervals during the night. The intakes that were achieved can therefore be considered a reflection of appetite, a variable that may be interpreted in terms of the natural history of convalescence from a severe burn.

A detailed report on food analyses has been presented elsewhere (13). Storage facilities for food were available for studies of 6 to 8 weeks' duration. Sample diets, prepared and cooked in the same manner in which they were served to patients, were analyzed periodically. Each of the menus was analyzed three to six times from each lot of food. Since it was demonstrated that the mineral content was significantly different in various lots of food, the mean of the analyses of a particular lot was used for calculation of the intake during the time when this lot was served. The composition of the formula was determined from the mean of repeated analyses. The volume of formula ingested was carefully measured. Uneaten foods (rejects) were pooled for individual metabolic periods and then analyzed.

Salt was not used for cooking, and the patients salted their own food from a salt shaker containing a carefully weighed amount of anhydrous sodium chloride. Food was served under close supervision of a member of the staff to avoid careless spillage of salt.

III. *Collection, preparation, and analysis of specimen*

The intake and urinary excretion of all minerals were measured in 24-hour metabolic periods for the first 2 to 3 weeks and in 5-day metabolic periods thereafter. Stools were pooled in 10-day periods from the beginning of the study. Dressings were pooled in convenient periods, the length of which was determined by the exigency of treatment in particular patients. During the early parts of a study, an effort was made to time dressing changes according to metabolic periods, but this practice proved too cumbersome and therefore had to be abandoned.

Glacial acetic acid, 50 ml., was added to the urine collection bottles at the beginning of each 24-hour collection period. Carmine was used as a marker for stool collection periods. Cascara tablets or enemas were used at the end of stool collection periods, and a distinct separation of stools into periods was usually possible. For unknown reasons, recovery of the carmine proved difficult in the early postburn period even though large quantities of carmine were given. When distinct stool separations were not feasible, the first stool collection period was extended to 20 days and occasionally even longer. The dressings were thoroughly washed in large washing machines, and aliquots of the washings were analyzed.

All samples were prepared and analyzed by methods that have been previously described (13).

To test the adequacy of the metabolic regimen in general, three normal subjects were observed for four 5-day periods each. They were found to be essentially in equilibrium with respect to all constituents studied.

IV. *Computation of balances and method of charting*

Balances were computed from all sources of intake and output. Electrolyte solutions, which were given in large volumes during the early postburn period, were not analyzed, and the composition as given by the manufacturer (volume and concentration) was used in calculating the intake from this source. Since plasma proteins enter the metabolic pool more rapidly than erythrocyte protein, nitrogen intake derived from whole blood transfusions was computed on the basis of the plasma protein content only; erythrocyte nitrogen was not counted as part of the intake. For estimating the mineral content of the plasma portion of whole blood, average values were used (7). Lyophilized plasma was given only rarely. The amount of whole blood given to the various patients is recorded on the metabolic correlation charts and in the Appendix. Since erythrocytes were not included in the intake, any dressings containing appreciable amounts of blood were discarded. This practice resulted in failure to measure some of the plasma proteins and minerals lost with the blood into the dressings. No allowance was made for 20 to 30 ml. of blood that were drawn daily in the first 2 to 3 weeks for a study of plasma electrolyte concentration changes. These studies will be reported separately.

For ease of interpretation, all of the data were charted on an average per day basis. The same grouping of data is used for all patients in order that a time-correlated comparison of their respective metabolic adjustment can be made visually. The day of injury is "day zero," the day after injury is the first postburn day, and so forth. The first 10 days of study are divided into a zero through second day period and a third through ninth day period. Thereafter, the balances are charted in 10-day intervals. The pooling of balances into arbitrary periods is a matter of convenience for graphic presentation. It obscures some important details, but these can be obtained from the original data.[2] Nitrogen, potassium, magnesium, and phosphorus are charted approximately in the relative proportions in which these minerals are believed to be present in normal muscle. Calcium and phosphorus are charted in a ratio that approximates the calcium-phosphorus ratio of bone. Sodium and chloride are charted separately. The intake is charted from the zero line up and the output from the top of the intake line down. Solid black areas indicate negative balance below the zero line and positive balance above it. The cross-hatched areas represent exudate contribution to the output. The relative contributions of urine and feces to the output are not shown.

Four ordinate scales are applicable to the five minerals charted on the upper part of the charts. One gram of nitrogen is charted as equivalent to 3.3 mEq. of potassium and 1/15 gm. of phosphorus. Calcium and phosphorus are charted in a ratio of 2:1. For charting purposes, these ratios are convenient approximations to the

[2] Detailed data are available to the reader on request: Lt. Col. Curtis P. Artz, Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Texas.

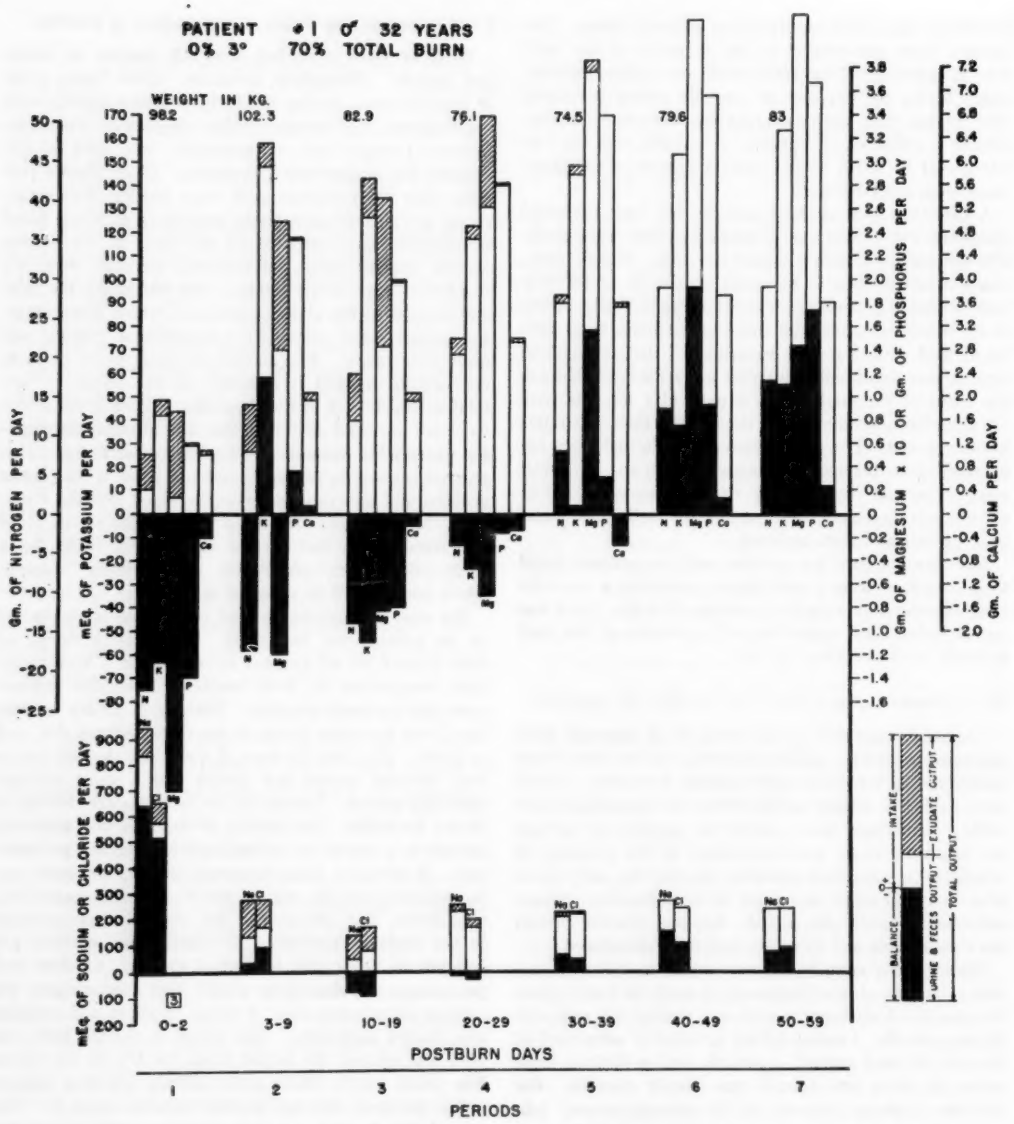

FIG. 1. METABOLIC CORRELATION CHART, PATIENT 1

- ● Skin graft.
- ① Two dressing changes under general anesthesia.
- ⊡ One unit of blood transfusion.

factors given by Reifenstein, Albright, and Wells (14). In Table I, the conversion factors recommended by these authors were used in computing theoretical balances, except that 3 mEq. rather than 2.7 mEq. of potassium were considered equivalent to 1 gm. of nitrogen. The conversion factor for intracellular magnesium is not well established. According to Baldwin, Robinson, Zierler, and Lilienthal (15) 0.61 mEq. (7.3 mg.) of magnesium correspond to 1 gm. of non-collagenous muscle nitrogen. Since 1/15 gm. (67 mg.) of phosphorus corresponds to 1 gm. of intracellular nitrogen, it was considered adequate for purposes of visual representation to use the same ordinate scale for "magnesium times 10" as for "phosphorus."

RESULTS

I. *Balances and Correlations*

Nitrogen balance

In patients 1 through 5, the duration of negative nitrogen balance (catabolic phase) was 28, 36, 31, 30, and 15 days, respectively (Figures 1–6). Negative nitrogen balance resulted from the very large excretion of urinary nitrogen as well as from a low dietary intake. The urinary nitrogen was much higher in the four men than in the woman (patient 4). There were large fluctuations from day to day in the amount of urinary nitrogen excreted, and the excretion rate tended toward a maximum at the end of the first postburn week. As the nitrogen balance approached equilibrium and became positive, the urinary nitrogen was generally low and, occasionally, exceptionally low. In patient 4, for example, the average urinary nitrogen was 5.9 gm. on an intake of 10.3 gm. 45 to 49 days after injury. The greatest urinary nitrogen loss on any one day occurred in patient 5, who excreted 36.9 gm. of nitrogen on the seventh postburn day on an intake of 18.8 gm. of nitrogen. Urinary nitrogen excretion of 25 to 30 gm. per day was commonly

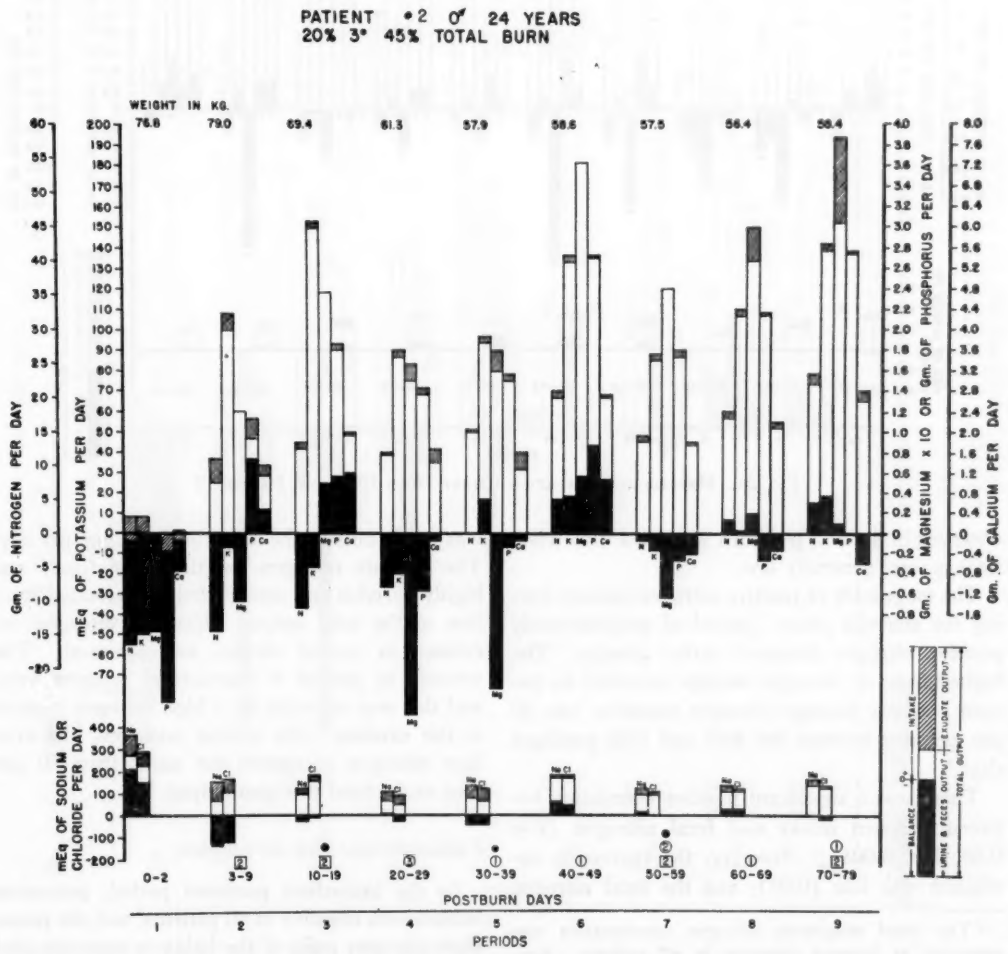
PATIENT • 2 ♂ 24 YEARS
20% 3° 45% TOTAL BURN
WEIGHT IN KG.
76.8 79.0 69.4 61.3 57.9 58.6 57.5 56.4 58.4
Gm. OF NITROGEN PER DAY
mEq OF POTASSIUM PER DAY
Gm. OF MAGNESIUM x 10 OR Gm. OF PHOSPHORUS PER DAY
Gm. OF CALCIUM PER DAY
mEq OF SODIUM OR CHLORIDE PER DAY
0-2 3-9 10-19 20-29 30-39 40-49 50-59 60-69 70-79
POSTBURN DAYS
1 2 3 4 5 6 7 8 9
PERIODS
INTAKE
EXUDATE OUTPUT
URINE & FECES OUTPUT
TOTAL OUTPUT
BALANCE

FIG. 2A. METABOLIC CORRELATION CHART, 0 TO 79 DAYS, PATIENT 2

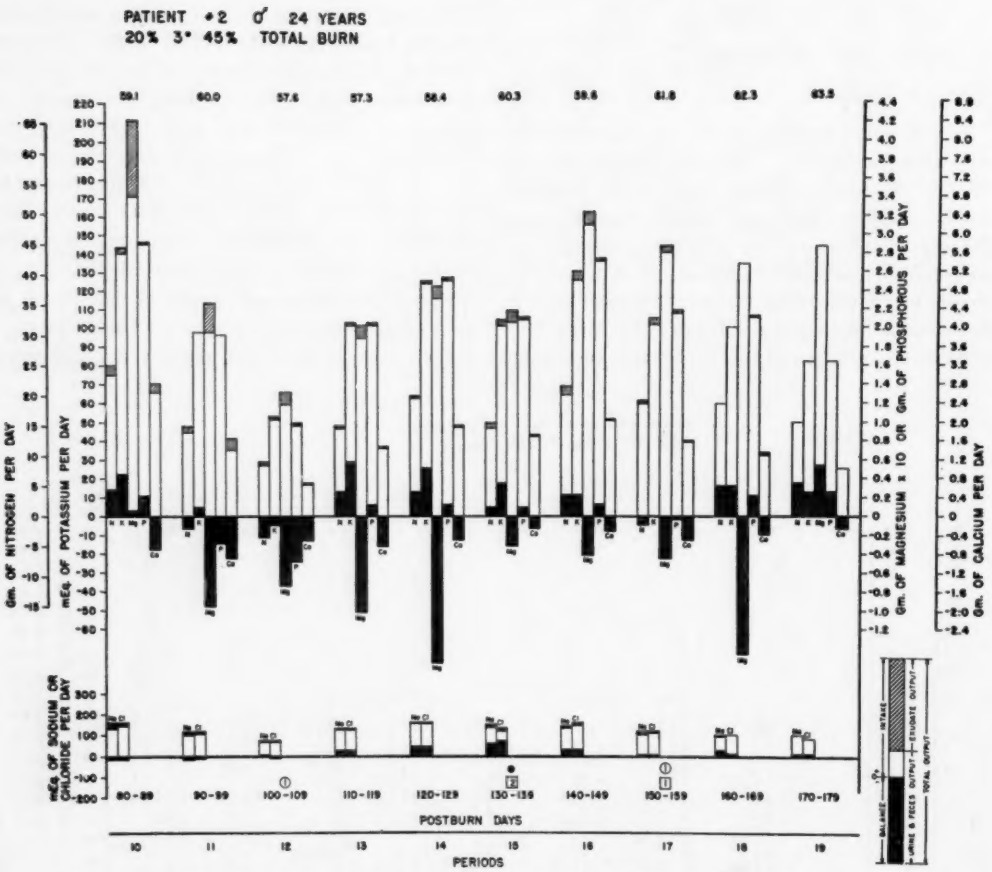

FIG. 2B. METABOLIC CORRELATION CHART, 80 TO 179 DAYS, PATIENT 2

observed in the first postburn week at a time when intakes were generally low.

The magnitude of positive nitrogen balance during the anabolic phase (period of predominantly positive nitrogen balance) varied greatly. The highest rate of nitrogen storage occurred in patient 1 whose average nitrogen retention was 20 gm. per day between the 46th and 55th postburn days.[3]

There was a significant positive correlation between nitrogen intake and fecal nitrogen ($r = 0.58$; $P < 0.001$). However, the regression coefficient was low (0.05), and the fecal nitrogen was rarely outside the range of 1 to 2 gm. per day. The exudate nitrogen, on the other hand, was highly variable and contributed a substantial fraction to the total output. Exudate nitrogen decreased as wound closure was achieved. The wounds of patient 4 deteriorated progressively, and this was reflected by a high nitrogen content in the exudate. On several occasions, the exudate nitrogen accounted for more than 50 per cent of the total nitrogen output.

Potassium-nitrogen correlation.

In the immediate postburn period, potassium balance was negative in all patients, and the potassium-nitrogen ratio of the balances approximated the value expected by whole tissue catabolism.

[3] The blood nonprotein nitrogen concentration was measured at frequent intervals in all patients. Azotemia was not observed at any time.

This early potassium excretion was followed by relative potassium retention, which is defined as less potassium excretion than would be expected if the negative nitrogen balance reflected whole tissue breakdown. Relative potassium retention occurred irrespective of the level of potassium intake. In the latter part of the catabolic phase, potassium balance again tended toward the expected value. In patients 2, 3, and 4, potassium balance was somewhat less negative than expected by nitrogen balance; in patient 1 it was more negative. The difference in response can be explained by reference to Figure 6, where it is seen that the unusually large amounts of potassium given to patient 1 in the early postburn period were temporarily retained in the body and excreted during the late catabolic phase.

In the anabolic phase, potassium balance was rather well correlated with nitrogen balance. Although marked fluctuations occurred between various periods and various patients, deviations from the expected ratio were not consistent either in magnitude or direction.

Large quantities of potassium were lost via the exudate. Except for relatively minor fluctuations, exudate potassium declined as wound closure proceeded. In patient 4, serious deterioration of the status of the wounds in late stages of the study

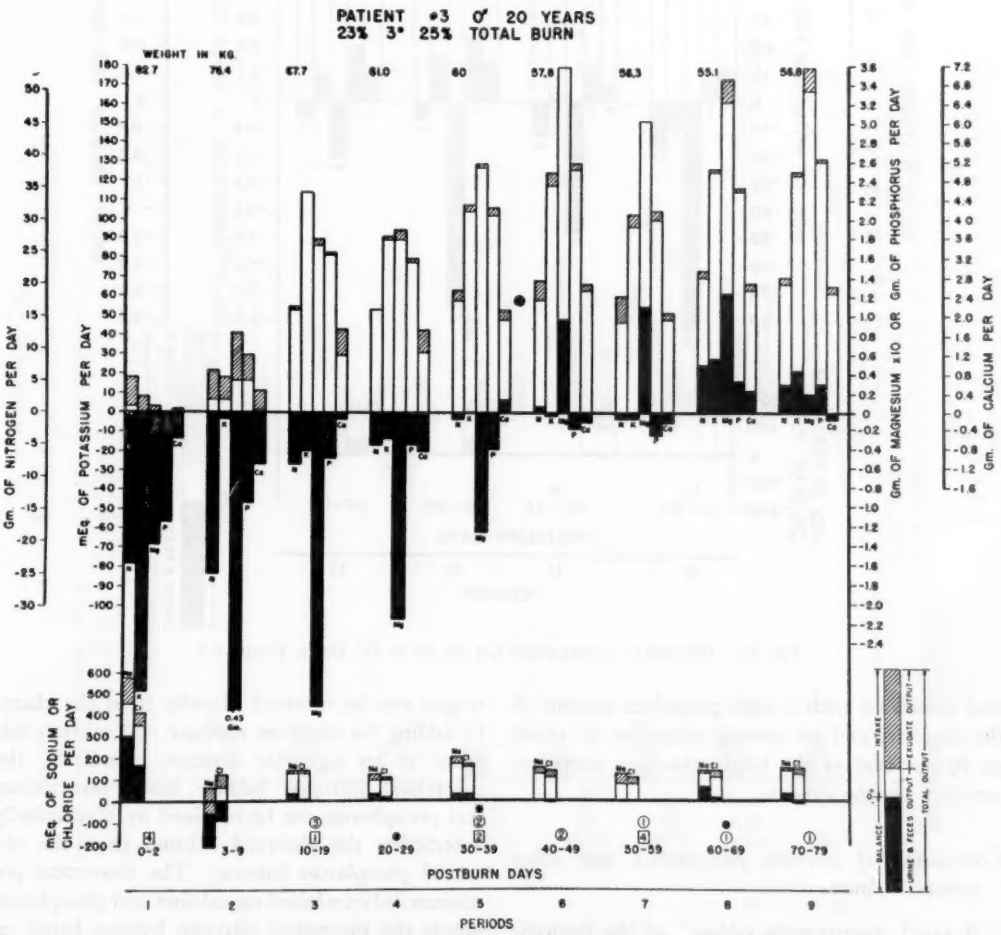

FIG. 3A. METABOLIC CORRELATION CHART, 0 TO 79 DAYS, PATIENT 3

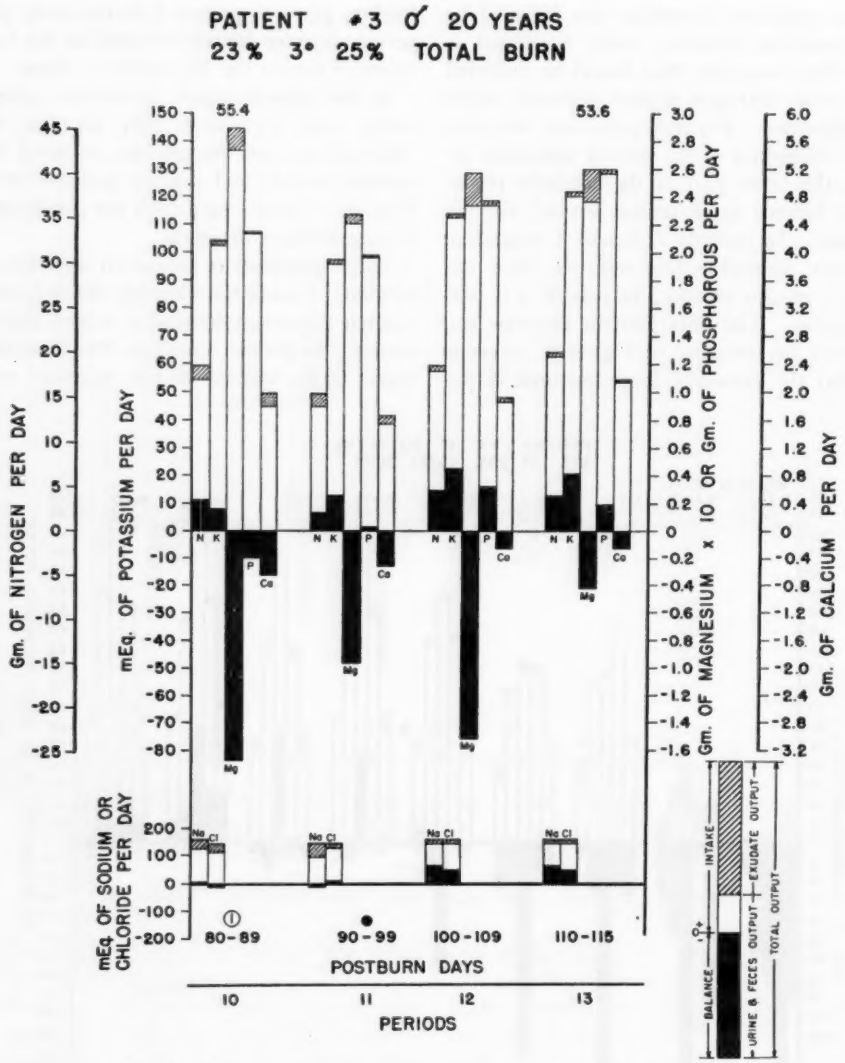

FIG. 3B. METABOLIC CORRELATION CHART, 80 TO 115 DAYS, PATIENT 3

was associated with a high potassium content of the exudate, and on several occasions as much as 40 per cent of the total potassium excretion occurred via the exudate.

Correlation of calcium, phosphorus, and other mineral balances

A rapid, approximate estimate of the theoretical phosphorus balance based on calcium and nitrogen can be obtained visually from the charts by adding the observed nitrogen and calcium balances in an algebraic manner. Similarly, the theoretical nitrogen balance based on calcium and phosphorus can be obtained by algebraically subtracting the observed calcium from the observed phosphorus balance. The theoretical potassium balance based on calcium and phosphorus equals the theoretical nitrogen balance based on these constituents.

In the early postburn period, the theoretical phosphorus balance based on calcium and nitrogen consistently deviated from the observed phosphorus balance, so that the observed phosphorus loss was smaller than that predicted on the basis of the nitrogen and calcium balances. During the same interval, the theoretical potassium balance based on nitrogen deviated markedly and consistently from the observed potassium balance so that the observed potassium excretion was less than that predicted on the basis of the nitrogen balance. Thus, the deviations from the expected values of both the phosphorus and potassium balances coincided in time as well as direction. In subsequent periods, there was, in general, agreement between the theoretical and observed phosphorus balances, both at times when nitrogen balance was positive and when it was negative. During many of the study periods, the theoretical phosphorus balance was in better agreement with the observed potassium balance than with the observed nitrogen balance.

Magnesium balance and correlation

In order to establish a baseline for comparison, three normal subjects were studied for four 5-day metabolic periods each. The intake was constant at approximately 26 mEq. of magnesium per day. On the average, 25 per cent of the ingested magnesium was excreted in the urine, the remainder

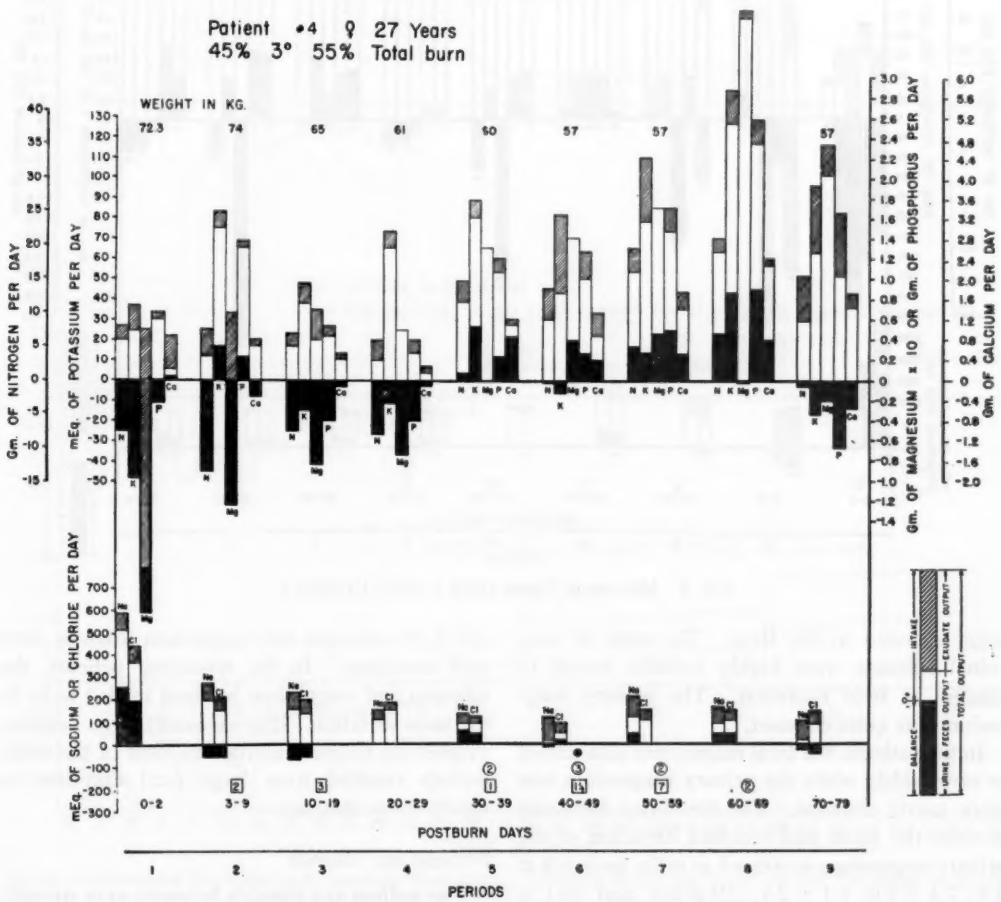

FIG. 4. METABOLIC CORRELATION CHART, PATIENT 4

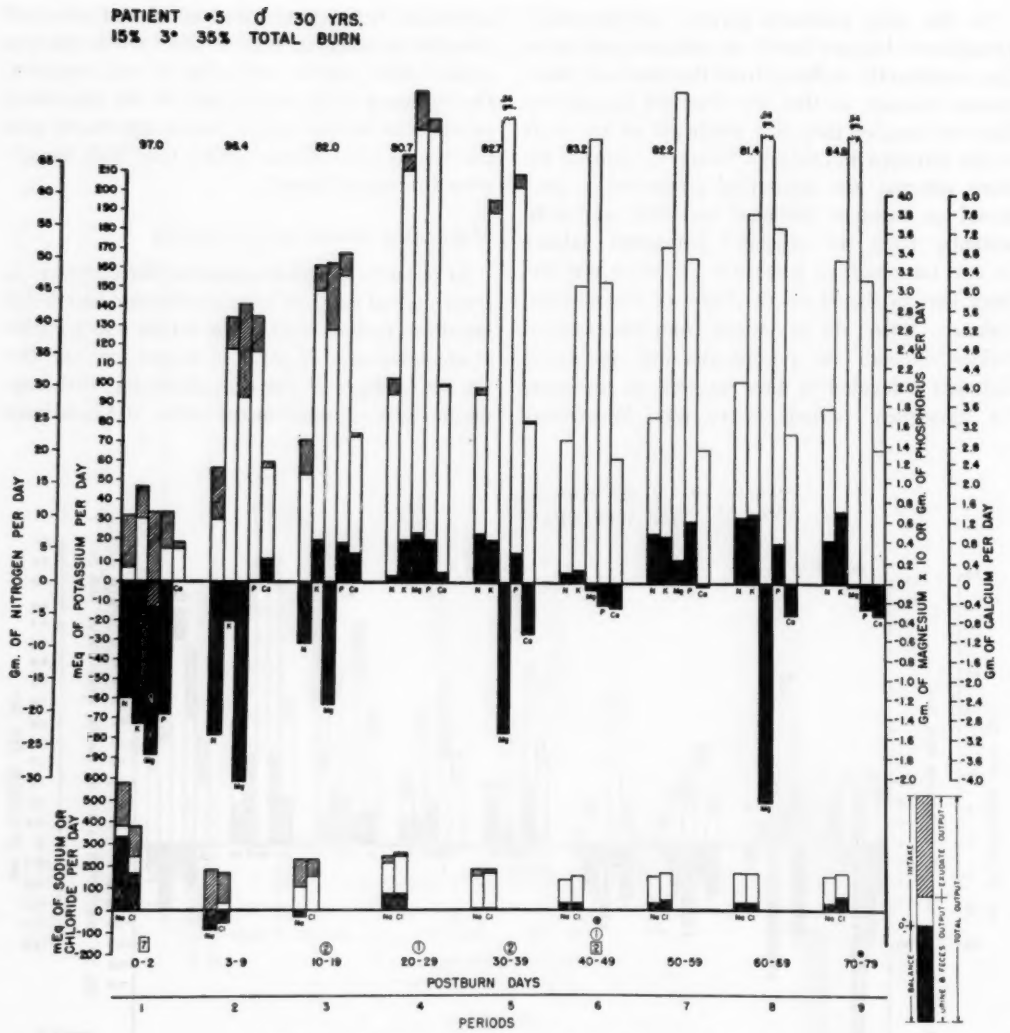

FIG. 5. METABOLIC CORRELATION CHART, PATIENT 5

being excreted in the feces. The data in individual subjects were highly variable owing to changes in fecal excretion. The urinary magnesium was quite constant.

In the patients, the fecal magnesium also tended to vary widely while the urinary magnesium was more nearly constant. For the 5-day metabolic periods, the mean and standard deviation of the urinary magnesium expressed as mEq. were 6.8 ± 3.3; 7.4 ± 2.0, 5.1 ± 2.1, 2.9 ± 0.9, and 10.1 ± 2.1 for the 5 patients, respectively. In patients 1 and 4, the nitrogen and magnesium balances were well correlated. In the remaining patients, the nitrogen and magnesium balances tended to be in the same direction. The unusually large negative magnesium balance recorded in some of the study periods resulted from large fecal excretion in nearly every instance.

Sodium and chloride

The sodium and chloride balances were strongly positive in the early postburn period. These posi-

tive balances are a result of the large amounts of these ions given in an effort to keep pace with the obligatory edema that is characteristic of recent burns. The exudate always contributed a large and important fraction of the total sodium and chloride output during this early period, and failure to take account of this important route of electrolyte excretion would result in paradoxically large positive balances. The loss of these electrolytes through the sweat was large in all of the patients. Burned patients are more comfortable in warm than in moderately cool surroundings. Air-conditioning, which was available, was poorly tolerated and could be used only rarely.

II. *Changes in Body Composition*

Whole tissue

If the early negative nitrogen balance is a reflection of whole tissue catabolism, the magnitude of whole tissue loss was very great in some of the patients. If 1 gm. of nitrogen is considered equivalent to 30 gm. of tissue, the patients lost between 8.2 kg. and 11.7 kg. of lean body tissue

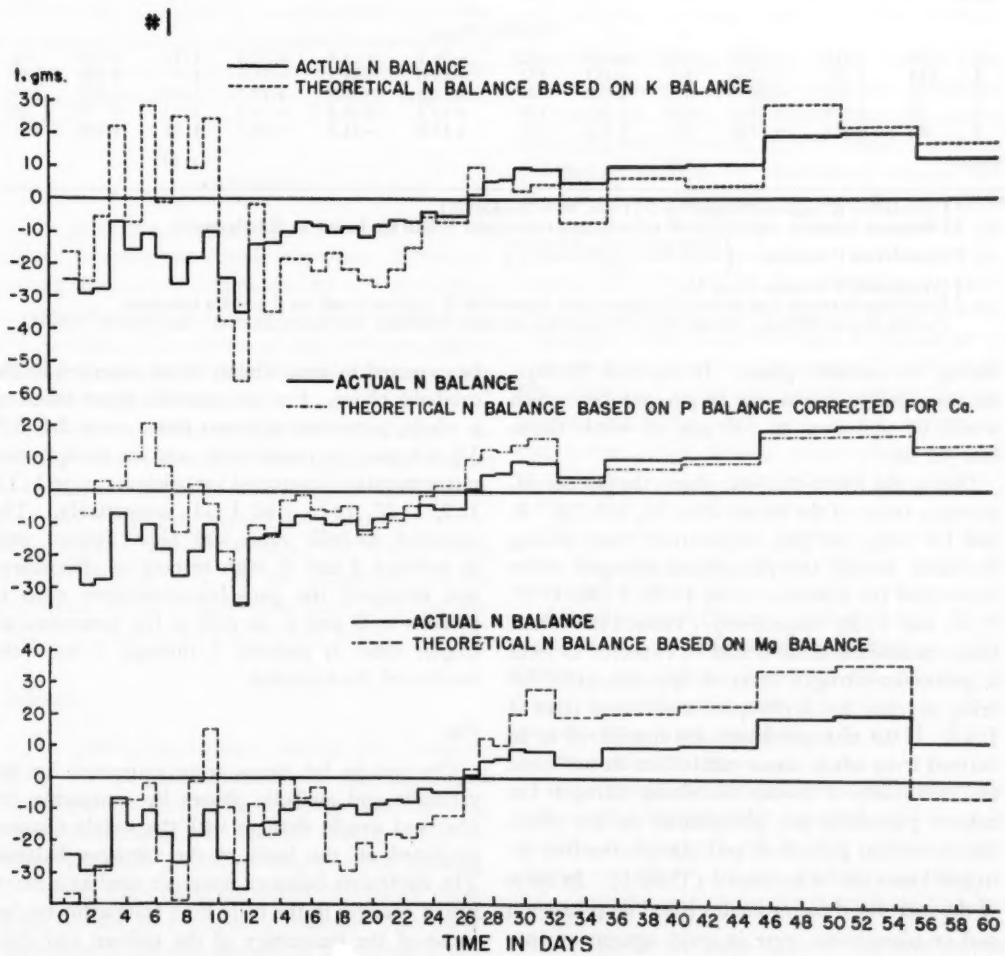
ACTUAL N BALANCE
THEORETICAL N BALANCE BASED ON K BALANCE
ACTUAL N BALANCE
THEORETICAL N BALANCE BASED ON P BALANCE CORRECTED FOR Ca.
ACTUAL N BALANCE
THEORETICAL N BALANCE BASED ON Mg BALANCE
TIME IN DAYS

FIG. 6. COMPARISON BETWEEN THE OBSERVED THEORETICAL NITROGEN BALANCES BASED ON THE BALANCES OF POTASSIUM, PHOSPHORUS AND CALCIUM, AND MAGNESIUM (PATIENT 1)

Note the early, relative retention of potassium and phosphorus.

TABLE I

Cumulative observed and theoretical nitrogen balances during catabolic and anabolic phases

Patient	Duration of phase	Potassium: Cumulative N balance	Potassium: Cumulative K balance	Potassium: K/N ratio	Potassium: * Theoretical N balance	Potassium: † Discrepance	Phosphorus: Cumulative P balance	Phosphorus: Cumulative Ca balance	Phosphorus: ‡ Protoplasm P balance	Phosphorus: Protoplasm P/N ratio	Phosphorus: § Theoretical N balance	Phosphorus: ‖ Discrepance
	days	*gm.*	*mEq.*	*mEq./gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*		*gm.*	*gm.*
					Catabolic Phase							
1	28	−390	− 830	2.1	−277	113	−13.4	− 6.4	−10.5	1:37	−158	232
2	36	−341	− 660	1.9	−220	121	− 7.2	+ 3.8	− 8.9	1:38	−134	207
3	31	−383	−1010	2.6	−337	46	−18.6	−18.6	−10.3	1:37	−154	229
4	30	−275	− 290	1.0	− 97	178	− 7.1	− 2.7	− 5.9	1:47	− 88	187
5	15	−322	− 440	1.4	−147	175	− 7.2	+ 3.9	− 8.9	1:36	−134	188
Mean				1.8		126				1:39		209
					Anabolic Phase							
1	33	+415	+1000	2.4	+333	(82)	+34.1	+ 3.4	+32.6	1:13	+489	74
2	144	+335	+1760	5.2	+587	252	+13.6	−54.1	+37.9	1:9	+568	233
3	85	+293	+1020	3.5	+340	47	+ 2.5	−18.2	+10.7	1:27	+160	(133)
4	58	+108	+ 730	6.8	+243	135	+12.1	+16.4	+ 4.7	1:23	+ 70	(38)
5	65	+347	+1720	5.0	+573	226	+19.8	−24.3	+30.7	1:11	+460	133
Mean				4.6						1:17		

* Cumulative K balance divided by 3 (1 gm. N = 3 mEq. K).
† Difference between cumulative N balance and theoretical N balance based on K balance.
‡ Cumulative P balance $- \left(\frac{\text{Cumulative Ca balance}}{2.23} \right)$.
§ Protoplasm P balance times 15.
‖ Difference between cumulative N balance and theoretical N balance based on P and Ca balances.
Figures in parentheses indicate that the expected nitrogen retention was approximated. See text for details.

during the catabolic phase. In the first 10 days, the mean nitrogen loss was 18 gm. per day, which would be equivalent to 540 gm. of whole tissue loss per day.

During the entire catabolic phase, the potassium-nitrogen ratios of the losses were 2.1, 1.9, 2.6, 1.0, and 1.4 mEq. per gm., respectively, and, during the same period, the phosphorus-nitrogen ratios (corrected for calcium) were 1:37, 1:38, 1:37, 1:47, and 1:36, respectively (Table I). Whole tissue catabolism alone would be expected to yield a potassium-nitrogen ratio of approximately 3.0 mEq. per gm. and a phosphorus-nitrogen ratio of 1:15. If the nitrogen losses are considered to be derived from whole tissue catabolism on one hand and catabolism of tissues containing nitrogen but neither potassium nor phosphorus on the other, the theoretical potassium and phosphorus-free nitrogen losses can be estimated (Table I). In some of the patients, these estimates based on potassium and on phosphorus were in good agreement (patients 4 and 5), while in others the agreement was poor.

In the anabolic phase, the mineral ratios could be expected to approximate those observed in the catabolic phase. For the anabolic phase taken as a whole, potassium-nitrogen ratios were 2.4, 5.2, 3.5, 6.8, and 5.0, respectively, and the phosphorus-nitrogen ratios (corrected for calcium) were 1:13, 1:9, 1:27, 1:23, and 1:11, respectively. The expected anabolic ratio was approximated only in patients 3 and 4, with respect to phosphorus and nitrogen; the phosphorus-nitrogen ratio in patients 1, 2, and 5, as well as the potassium-nitrogen ratio in patients 2 through 5 were the reverse of the expected.

Fat

Changes in fat stores were estimated for the catabolic and anabolic phases by comparing the observed weight changes with the weight changes predicted on the basis of the nitrogen balance. The electrolyte balances were not used as a measure of changes in the body fluid compartments because of the inaccuracy of the sodium and chloride balances that resulted from sweat loss. In order to avoid the cumulative errors in the electrolyte balance, the two weights used for calculating

the weight change were chosen so that they coincided with a time when the patients were in normal fluid balance as judged by clinical criteria. This could be readily accomplished at the end of the catabolic phase and at an appropriate time chosen during the anabolic phase. Since the patient's preburn weight was not always accurately known, the preburn weight was determined from the first weight determination performed on the ward (usually within the first 24 hours after burning) corrected for the fluid balance from the time of injury until the time of the first weight determination.

The data in Table II were calculated on the assumption that 1 gm. of nitrogen is equivalent to 30 gm. of tissue. Substantial fat losses appear to have occurred in both the catabolic and anabolic phases.

TABLE II

Estimated body fat changes during the catabolic and anabolic phases

Patient	Period	N balance	Theoretical wt. change based on N	Actual wt. change	Discrepance
	days	*gm.*	(N×30) ÷1000 *Kg.*	*Kg.*	*Kg.*
1	0–27	−390	−11.7	−15.8	4.1
	28–60	+415	+12.4	+ 6.6	5.8
2	0–35	−341	−10.2	−14.1	3.9
	36–179	+335	+10.0	+ 5.6	4.4
3	0–30	−383	−11.5	−19.0	7.5
	31–115	+293	+ 8.8	− 5.5	14.3
4	0–29	−275	− 8.2	−12.3	4.1
	30–87	+108	+ 3.2	− 3.6	6.8
5	0–14	−322	− 9.6	−10.2	0.6
	15–79	+347	+10.4	+ 5.8	4.6

DISCUSSION

The problems inherent in metabolic studies in burned patients are readily apparent. Foremost among these are the impossibility of maintaining a constant intake and the necessity for measuring the exudate. Hemolysis of considerable degree during the postburn period introduces a variable in the nitrogen balance that cannot be assessed. Treatment necessitates frequent transfusions at varying intervals of time throughout the course of illness, and failure to include red cell nitrogen as part of the nitrogen intake causes a sizable cumulative error. These difficulties are compensated for, at least in part, by the magnitude of the metabolic changes that take place and by the length of observation used in this study.

Balances

The potassium balance during the catabolic phase is of particular interest and may be divided into three periods: 1) Immediately after burning, potassium balance was negative. This was associated with low potassium intakes in all patients. 2) Within a few days, the extent of negative potassium balance was markedly lessened, and potassium balance became positive at a very early time if liberal amounts of potassium were given. 3) In the later part of the catabolic phase, potassium balance became negative again. At this time, potassium balance appeared to be less influenced by potassium intake, and large potassium intakes were frequently associated with negative balance.

Interpretation of the balances of calcium and phosphorus in the absence of constant intakes of these minerals is difficult, since changes in the intake are only slowly reflected in the fecal excretion (fecal lag). In the present study, pronounced fluctuations in the intake were inevitable. The highly variable contributions to the output by urine and feces can be seen in the detailed data.[2] The long fecal collection periods tended to compensate partly for fecal lag. In the correlation charts, the highly variable nature of calcium and phosphorus balance data becomes less apparent owing to the pooling of data.

Since preburn control balances are not available, the cumulative calcium balances cannot be compared with the studies on 6 to 7 weeks of immobilization that Deitrick, Whedon, and Shorr performed in healthy adult male subjects (16). There is no evidence, however, that the calcium losses in the burned patients were substantially greater than those expected as a result of prolonged immobilization.

The urinary calcium excretion was unusually high in all patients except patient 4. This was in general accompanied by a high urinary phosphorus excretion.[4] In the presence of such findings, there is a serious danger of renal calculus for-

[4] It is known that renal calcinosis and hypercalcemia with eventual renal failure are often associated with urine calcium levels of this magnitude. Plasma calcium levels and renal functions were not measured in these patients.

mation.[5] Although the incidence of renal calculi appears to be small, the advisability of giving diets with a high calcium and phosphorus content is open to question in view of the findings presented here.

Changes in the magnesium balance reflected, to a large extent, changes in fecal magnesium excretion. The latter was distinctly influenced by the level of magnesium intake. It was of interest that, in several study periods, the fecal magnesium was greater than the magnesium intake. This indicates either that magnesium was excreted into the gastrointestinal tract or that fecal excretion of previously ingested magnesium was greatly delayed. Strongly positive magnesium balance was observed only in patient 1 during the latter part of the study. This resulted from a slightly decreased fecal magnesium excretion that was associated with a greatly increased magnesium intake.

Correlations and changes in body composition

The most striking and consistent change in the balances was the failure of the early potassium and phosphorus balances to correlate with the nitrogen balance. When the entire catabolic phase is considered, the catabolized tissue contained less potassium and phosphorus in relationship to nitrogen than normal protoplasm. This may be explained as the loss of a relatively nitrogen-rich tissue or as loss of protoplasm with deposition of potassium and phosphorus, either in intracellular fluid or with glycogen. Since collagen and skin contain a large amount of nitrogen in relation to potassium, lysis of large amounts of these tissues may account for part of the nitrogen lost in excess of that expected by the potassium balance. Catabolism of plasma proteins, liver proteins, and other reserve protein stores may explain part of the large nitrogen losses in excess of potassium and phosphorus during the early catabolic phase.

The ratio of nitrogen to other intracellular constituents during the anabolic phase would be expected to be the same as that during the catabolic phase. Actually, these ratios were often the reverse of the expected during the anabolic phase. This may be explained partly by the fact that the catabolic phase was studied in its entirety while the anabolic phase certainly extended far beyond the completion of these studies. None of the patients had achieved nitrogen equilibrium by the time the studies ended. When patients 1 and 5 were discharged from the hospital, the nitrogen losses incurred after injury had been replaced in both patients so that the cumulative nitrogen balance from the time of injury to discharge approached zero. Two months after discharge, additional studies were performed in these patients for four 5-day metabolic periods. At that late date, both patients were still in significantly positive nitrogen, potassium, phosphorus, calcium, and magnesium balances. Thus, convalescence in these patients was characterized by mineral retention in excess of the amounts present in the body before injury. This is most surprising since the capacity for mineral storage of healthy adults is quite limited.

Clinical data

During the catabolic phase, areas of partial-thickness skin loss healed satisfactorily in all patients, and skin grafts took well in patients 2, 3, and 5, who were grafted during this period. During the anabolic phase, clinical correlations between nitrogen balance and a good healing capacity could not always be established. Positive nitrogen balance *per se* does not appear to insure proper healing, and other factors, such as degree of depletion of body proteins and fat stores, endocrine changes, and changes in the wound itself, probably play dominant roles in the healing process.

SUMMARY

1. The balances of nitrogen, potassium, phosphorus, calcium, magnesium, sodium, and chloride were measured in five severely burned patients from the time of injury to complete healing.

[5] In a review of 650 burned patients, most of whom were immobilized for at least three months and frequently much longer, there was one patient who had bilateral renal calculi, two patients who had a bladder calculi, and two patients in whom renal calculus formation was suspected clinically but not confirmed roentgenologically. The incidence of asymptomatic calculus formation in the genito-urinary tract is not known. Nearly all patients were given diets with high calcium and phosphorus contents similar to those used in the present study. The calcium and phosphorus contents of the diets are high because milk and milk products are used as supplements to the regular diet.

Individual studies varied in length from 60 to 180 days.

2. The nitrogen losses incurred during the catabolic phase could not be accounted for on the basis of whole tissue dissolution: the negative nitrogen balance was greater than could be explained by either the potassium balance or the phosphorus balance corrected for calcium.

3. In general, magnesium balance paralleled the nitrogen balance in direction, but the nitrogen-magnesium ratio of catabolized tissue and anabolized tissue varied greatly.

4. Body fat losses, estimated from nitrogen balance and weight changes, appeared to occur in all patients during both the catabolic and the anabolic phases.

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APPENDIX

Patient 1. This 32-year-old Negro airman received partial-thickness (second degree) burns of 70 per cent of the body surface when a relative attempted to kill him by pouring boiling water over him while he was sleeping in the nude. Before injury, the patient had been in excellent health.

Physical examination revealed a well-developed, well-nourished man who had burns involving the face, both upper extremities, portions of the anterior and posterior aspects of the trunk, and both thighs. Local care consisted of superficial debridement and cleansing of all burned areas, and the application of compression dressings over all areas except the face and the anterior aspect of the trunk; these were treated by the exposure method. During the first 48 hours after burning, the patient was given intravenously 1500 ml. of blood, 1500 ml. of plasma, 1000 ml. of 0.9 per cent saline, 9000 ml. of lactated-Ringer's solution, and 1000 ml. of 5 per cent dextrose in water. On this regimen, the urinary output was maintained at approximately 50 ml. per hour, and signs and symptoms of circulatory collapse were not observed. Swelling of the wounds began immediately after injury and increased progressively until the 8th postburn day. Until this time, the patient's clinical course was characterized by progressive deterioration as evidenced by periods of disorientation, abdominal distention, and increasing fever (104° F. on fifth postburn day). On the 8th postburn day, a diuresis occurred, the urine volumes being 5180 ml., 5140 ml., 3600 ml., 5160 ml., and 6440 ml. on the 8th, 9th, 10th, 11th, and 12th postburn days, respectively. Concomitantly, the sensorium cleared, abdominal distention ceased, and the temperature became normal. Thereafter, the patient felt well and ingested increasing quantities of food.

The wounds healed satisfactorily so that most burned areas were epithelized within 3 weeks. Complete healing was noted on the 33rd postburn day. Convalescence was complicated on the 22nd postburn day by what was believed to be phlebothrombosis of the leg veins and a small pulmonary infarct. Heparin treatment was given. The patient was ambulatory on the 35th postburn day. He returned to the hospital for follow-up studies 106 days after injury. At that time, his weight had returned to preburn levels.

Patient 2. This 24-year-old white airman received extensive burns when a kerosene stove exploded in his trailer. The patient's wife was fatally burned in the same accident. Before injury, the patient had been in good health except for minor psychiatric difficulties.

Physical examination revealed a well-developed, well-nourished man who had burns extending over 45 per cent of the body surface. Full-thickness (third degree) burns were noted on the face, the scalp, and both upper extremities, a total of 20 per cent of the body surface. Partial-thickness burns involved the trunk and the anterior aspect of the thighs. Local care consisted of superficial debridement and cleansing of all burned areas, exposure treatment of the face, and compression dressings over the other burned areas. A tracheotomy was performed a few hours after injury.

Supportive therapy during the first 48 hours consisted of 750 ml. of plasma, 1300 ml. of 0.9 per cent saline, 5000 ml. of lactated-Ringer's solution, and 1000 ml. of 5 per cent dextrose in water. On this regimen, the urinary output was maintained at approximately 40 ml. per hour, and signs and symptoms of circulatory collapse did not develop. Fever (105° F.) became evident on the 5th day postburn, and on the 9th postburn day, a diagnosis of septicemia caused by Micrococcus-pyogenes, var. aureus, coagulase positive, was established by blood culture. Therapy consisted of Chloramphenicol intravenously. The temperature gradually receded to between 100° F. and 101° F., and the last positive blood culture was obtained on the 12th postburn day.

The patient was disoriented immediately after injury and had episodes of disorientation and hallucinations for the first month. It was believed that these were caused by his inability to see (edema of lids and, later, tarsorrhaphy) and his concern about his wife's death and his disfigurement, which accentuated psychiatric difficulties that were present before injury.

The partial-thickness burns were healed within a month after injury. Many skin grafts failed to take ow-

ing to excessive wound suppuration. Convalescence was complicated by homologous serum hepatitis. This was diagnosed on the basis of jaundice and liver function tests on the 95th postburn day and delayed further skin grafting.

Skin grafts: 16th, 32nd, 59th, and 136th postburn days. General anesthesia for dressing changes: 22nd, 28th, 29th, 38th, 43rd, 50th, 57th, 63rd, 72nd, 109th, and 156th postburn days. Blood transfusions: 5th (2 units), 15th (1 unit), 16th (1 unit), 59th (2 units), 73rd (2 units), 136th (2 units), and 156th (1 unit)—a total of 11 units.

Patient 3. This 20-year-old white airman received burns over 25 per cent of the body surface when a transport plane, in which he was a passenger, crashed after take-off. Before injury, the patient had been in excellent health.

Physical examination revealed a well-developed, well-nourished man who had deep full-thickness burns of the face, both upper extremities, both thighs, and the anterior aspect of the trunk. Full-thickness burns extended over 23 per cent of the body surface and partial-thickness burns over 2 per cent. After superficial debridement and cleansing, compression dressings were applied to all burned areas except the face, which was treated by the exposure method. A tracheotomy was performed a few hours after injury.

During the first 48 hours, 500 ml. of plasma, 2000 ml. of blood, 500 ml. of dextran, 1000 ml. of Ringer's solution, 3000 ml. of 0.9 per cent saline, 1850 ml. of lactated-Ringer's solution, and 3450 ml. of 5 per cent dextrose in water were given as supportive therapy. This large volume of fluids, which in retrospect appears excessive, was given because the extent of burn was overestimated initially. The urinary output was in excess of 150 ml. per hour on many occasions during the first 48 hours; signs and symptoms of circulatory collapse did not develop. Septicemia caused by Alkaligenes faecalis and coagulase negative micrococci was diagnosed by blood culture on the 4th postburn day, a sudden temperature rise to 105° F. having aroused suspicion of this complication. On therapy with erythromycin, the blood culture became negative and the fever receded.

The patient's hospital course was complicated by repeated graft failures owing to Group A beta hemolytic streptococcal colonization of the wounds.

Skin grafts: 23rd, 32nd, 69th, and 96th postburn days. Dressing changes under anesthesia: 13th, 16th, 19th, 30th, 38th, 45th, 48th, 54th, 67th, 73rd, and 81st postburn days. Blood transfusions: 13th (1 unit), 31st (1 unit), 32nd (1 unit), 55th (1 unit), 68th (2 units), and 69th (2 units) postburn days. Total blood transfusions—8 units.

Patient 4. This 27-year-old Negress received extensive body burns when her clothes ignited as a result of her pouring kerosene on a hot stove. She had a normal, full-term infant two and a half months before the burning accident and had noted intermittent, scanty vaginal bleeding since the delivery.

Physical examination revealed a slightly obese woman who had burns involving 55 per cent of the body surface, including 45 per cent full-thickness burns and 10 per cent partial-thickness burns. Full-thickness skin loss extended over the neck, the anterior and posterior aspects of the trunk, and the thighs; partial-thickness skin loss extended over the hands and the face. Local care consisted of superficial debridement and cleansing of all burned surfaces, and the application of compression dressings over all burned areas except the face, which was treated by the exposure method.

Supportive therapy during the first 48 hours after injury consisted of the following fluids: 1000 ml. of blood, 500 ml. of plasma, 500 ml. of dextran, 2000 ml. of 0.9 per cent saline, and 6000 ml. of lactated-Ringer's solution. On this regimen, signs and symptoms of circulatory collapse did not develop. A urine flow of approximately 50 ml. per hour during the first 48 hours and thereafter a progressively increasing rate of urine flow gave evidence of satisfactory fluid redistribution. A blood culture, taken on the 4th postburn day because of fever of 103° F., was positive for Micrococcus pyogenes, var. aureus, coagulase positive. This microbe was sensitive *in vitro* to oxytetracycline, and treatment with this antibiotic, 2 gm. intravenously per day, resulted in blood stream sterilization within two days. Progressive salt and water deficits developed and escaped attention from the 6th to the 10th postburn days. When appropriate fluid therapy was given, acute gastric dilatation developed; this was treated by continuous gastric suction. From this time on, gastrointestinal atony was a problem, intermittently, throughout the patient's hospital course, and prevented the use of an adequate feeding regimen.

As a result of these complications, clinical management was made difficult. The partial-thickness burns healed satisfactorily, and grafts applied to the neck and the chest above the nipple line on the 41st postburn day took to the extent of 95 per cent. However, the donor sites (lower legs and upper arms) were converted, in large measure, to full-thickness skin loss. This conversion, which was believed to be a result of severe malnutrition, delayed further grafting. The clinical course was one of progressive deterioration. On the 86th postburn day, the patient had a cardiac arrest during induction of anesthesia. Cardiac massage was instituted and a normal sinus rhythm was resumed, but the patient did not regain consciousness and died within 12 hours. Skin graft: 41st postburn day. Dressing changes under anesthesia: 34th, 37th, 40th, 43rd, 48th, 54th, 58th, 61st, 69th, and 86th postburn days. Blood transfusions: 4th (1 unit), 5th (1 unit), 14th (1 unit), 15th (2 units), 16th (1 unit), 34th (1 unit), 41st (1½ units), 53rd (2 units), 54th (2 units), 55th (2 units), 59th (1 unit), 84th (2 units), 85th (2 units), and 86th (2 units) postburn days—a total of 21½ units.

Patient 5. This 30-year-old white airman received burns over 35 per cent of the body surface in a night-club fire. Before injury, the patient had been in excellent health.

Physical examination revealed a well-developed, well-nourished man who had full-thickness burns of both upper extremities (15 per cent of body surface) and partial-thickness burns of the face and the back (20 per

cent). Local care consisted of superficial debridement and cleansing of all burned surfaces, and the application of compression dressings over all burned areas except the face, which was treated by the exposure method.

Supportive therapy during the first 48 hours consisted of 3400 ml. of blood, 1500 ml. of lactated-Ringer's solution, 1000 ml. of 10 per cent invert sugar, and 300 ml. of 5 per cent dextrose in water. On this regimen, the urine flow was 75 ml. per hour on the average. Circulatory failure did not develop, but the patient was disoriented and restless during the first seven postburn days. On the fifth postburn day, fever of 105° F. suggested the presence of septicemia, but several blood cultures failed to establish this diagnosis. The temperature receded to 101° F. by the 7th postburn day. Concomitantly, the patient became oriented and cooperative.

The subsequent clinical course was uneventful except for wound suppuration and poor graft takes as a result of colonization of the wounds by Group A beta hemolytic streptococci. This accounts for the protracted course. The partial-thickness burns were well healed by the 25th postburn day, and nearly complete skin coverage was achieved by the 50th postburn day. Skin grafts: 43rd and 70th postburn days. Dressing changes under anesthesia: 10th, 13th, 29th, 34th, 37th, and 40th postburn days. Blood transfusion: 43rd postburn day (2 units).

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STUDIES IN CALCIUM METABOLISM. THE FATE OF INTRAVENOUSLY INJECTED RADIOCALCIUM IN HUMAN BEINGS [1, 2, 3]

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In the course of studies on the effect of phytates on calcium uptake in man (1) it became necessary to determine the extent to which endogenous calcium in the feces might contribute to the calcium balance. Although this point has been studied by several investigators (2–5), there is considerable disagreement on the significance of the fecal route for calcium excretion in man. Thus Malm (6) has observed that different individuals may exhibit pronounced variations in the fecal calcium excretion and has suggested that extended observations be made on each proposed test subject. Since this approach is not practical in many cases, it seemed desirable to establish the significance of the endogenous calcium output in the feces under our experimental conditions.[5]

The method chosen for investigation was to inject radiocalcium (Ca^{45}) intravenously and to determine its concentration in the serum, urine and feces during several days. Such studies have been carried out in animals (7–10), but few such data are available for man (11–13).

MATERIAL, DESIGN AND METHODS

Subjects. Nine adolescent boys, institutionalized for mental inadequacy, but otherwise normal,[6] served in the first study (Experiments A and B). In a later experiment (Experiment C), one individual (No. 57) was selected for intensive study. He was a spastic, but neither clinical nor laboratory examinations revealed any abnormalities in his mineral metabolism. The boys ranged in age from 10.9 to 15.7 years, with mean and median ages of 12.6 years. Their height ranged from 124 to 160 cm. with mean and median heights of 142 cm. Their weight ranged from 29.8 to 44.5 kgm., with a mean weight of 35.6 kgm., and a median weight of 36.0 kgm. The mental age of the boys averaged 7.2 years, (median: 7.2 years) and ranged from 5.8 to 9.2 years.

The young male adult in Experiment C was 21 years old, weighed 51.4 kgm. and had a mental age of 10.0 years.

Design. The subjects (Experiments A and B) were divided into two groups. One group (I.V.) was given the Ca^{45} intravenously, while the other group (P.O.) received Ca^{45} by mouth. One month later, the P.O. group received the intravenous injection, while the I.V. group received the isotope orally.[7] This was done to minimize the effect of periodic or seasonal variations (6, 14) and to use each individual as his own control. Preliminary tests had shown that one month after the administration of 0.7 μc Ca^{45}, the level of Ca^{45} in the blood and in the excreta was no longer measurable. Two years later (Experiment C), one individual was studied intensively

[1] A portion of this paper was presented at the XIX Physiological Congress, Montreal, Canada, August 31–September 4, 1953. This article is based in part on a dissertation presented by one of us (FB) in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Food Technology, Massachusetts Institute of Technology (1952).

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[5] When Dr. F. X. Aylward was a guest of the Massachusetts Institute of Technology, his friendly interest in our studies provided some of the original stimulus for establishing this point.

[6] All of the subjects were under medical care and observation during their usually indefinite institutionalization. They were selected on the basis of clinical examination. Roentgenographs of the chest, knees, hands, skull and spine of six subjects (Nos. 10, 11, 13, 14, 15, 16) were evaluated by an experienced roentgenologist, who concluded that they did not deviate from the normal range with respect to calcification.

[7] The results on the study with ingested Ca^{45} will be presented elsewhere.

with a larger dose of intravenously administered Ca^{45} under conditions similar to those which prevailed in Experiments A and B.

Dietary regime. All individuals ate the regular institutional diet, which was shown by analysis to meet the Recommended Dietary Allowance of the National Research Council (15) for subjects of this type. Beginning two weeks preceding, and continuing through the experiment, all subjects received daily an additional glass (240 ml.) of milk and one multi-vitamin tablet.[8] On the day of the experiment, the subjects ate a special test breakfast consisting of 180 gm. of cooked oatmeal (approximately 35 gm. rolled oats, 8.3 per cent moisture), and of 240 ml. whole milk. The total calcium and phosphorus contents of the breakfasts were, respectively, 329 and 370 mgm. in Experiment A, 294 and 295 mgm. in Experiment B, and 295 and 303 mgm. in Experiment C.

Radiocalcium.[9] The Ca^{45} was diluted in 0.85 per cent sodium chloride solution in a rubber-capped vial which was sterilized by autoclaving at 20 psi. for 20 min. Three milliliters of the solution were withdrawn from the vial into sterile 5 ml. glass syringes and injected into the antecubital vein of the subject approximately 30 minutes following the test breakfast. The dose given was 0.70 microcuries (152×10^3 counts/minute) in Experiment A, 0.74 microcuries (159×10^3 c/m) in Experiment B, and 2.02 microcuries (583×10^3 c/m) in Experiment C.

The accuracy of the dose measured in this way was tested by "injecting" a dose into a volumetric flask with the aid of a syringe and measuring the radioactivity of a suitably diluted aliquot. The quantity of Ca^{45} dispensed was found to be identical (within five per cent) to that calculated.

Serum and specimen collections

1. *Serum.* To reduce the number of venipunctures to a minimum, schedules of staggered collections of serum on the first day were worked out for the individuals in Experiments A and B. Thereafter, the sera were collected once daily for four days.

The serum samples in Experiment C were obtained nine times on day 1, once daily for four more days, then once weekly, and finally once monthly.

2. *Urine and feces.* Twenty-four-hour specimens of urine and feces were collected in glass jars daily for five days in Experiments A and B. In Experiment C, the collection period was extended beyond the first five days as follows: 48-hour specimens of urine and of feces were obtained on days 8–9, 15–16, 22–23, 29–30 and 58–59 following injection.

The urine was preserved by refrigeration and by the addition of glacial acetic acid, 2 per cent by volume. Feces were preserved under toluene.

Methods of analysis for Ca^{40} and Ca^{45}. The procedures employed have been described previously (1). In the analysis for Ca^{45} content, suitable standards were employed in all counting runs and the decay factors determined experimentally. All counting data were corrected for decay from the time of injection and are reported in terms of total activity (per cent of injected Ca^{45}) or in terms of specific activity (per cent of injected Ca^{45} per milligram calcium).

The error in replicate calcium determinations was usually held to 3 per cent standard deviation (S.D.); when samples were ashed before isolation of the calcium oxalate, the total error approximated 5 per cent S.D.

Counting errors varied according to experiment and tissue and were commonly held below 10 per cent S.D.

RESULTS AND DISCUSSION

Serum

The results of the measurements of the specific activity of the serum in Experiments A, B and C are presented in Figure 1. The values plotted here are the experimental values after adjustment to the mean body weight of the boys of Experiments A and B. For example, 56 minutes following the injection, the observed specific activity of subject No. 16 was 0.050 per cent of the injected Ca^{45} per milligram serum calcium. However, his body weight was 1.1 times that of the group average of 35.6 kgm. Since he had a proportionately larger serum calcium reservoir, the observed specific activity value was $\frac{1}{10}$ lower than it would have been if this boy had been smaller. Consequently the adjusted specific activity value was 0.055 per cent per mgm. Ca.

Because of the schedule of staggered venipunctures on Day 1 (Experiments A and B) the resulting plot is a composite of individual curves and not strictly comparable to the curve obtained on only one individual in Experiment C. At the same time it explains the comparatively wide scatter of the values of Experiments A and B.

As can be seen from Figure 1, the specific activity of the serum decreases monotonically as a function of time and shows no apparent tendency

[8] Vi-Penta® Perles were kindly furnished by Hoffman-LaRoche Inc., and were guaranteed to contain: vitamin A, 5000 U.S.P. units; vitamin B_1, 3 mgm.; vitamin B_2, 3 mgm.; vitamin B_6, 1 mgm.; panthenol, 3 mgm.; niacinamide, 20 mgm.; vitamin C, 75 mgm.; vitamin D, 1000 U.S.P. units; vitamin E acetate, 1 mgm.

[9] Obtained from the Oak Ridge Installation of the Atomic Energy Commission. Authorization for use of tracer doses of Ca^{45} in mentally disturbed patients was granted through the Sub-committee on Human Applications.

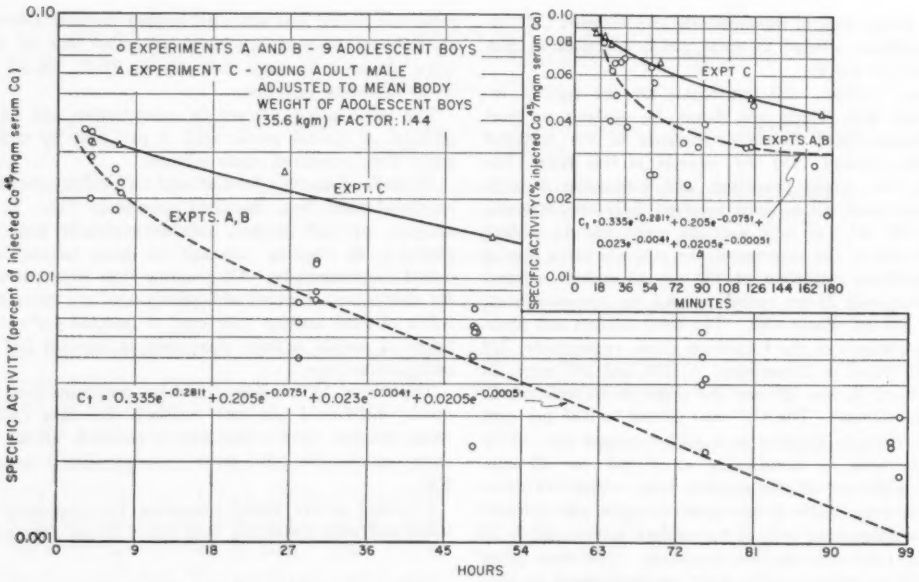
o EXPERIMENTS A AND B - 9 ADOLESCENT BOYS
Δ EXPERIMENT C - YOUNG ADULT MALE ADJUSTED TO MEAN BODY WEIGHT OF ADOLESCENT BOYS (35.6 kgm) FACTOR: 1.44
EXPT. C
EXPTS. A, B
$C_t = 0.335e^{-0.281t} + 0.205e^{-0.075t} + 0.023e^{-0.004t} + 0.0205e^{-0.0005t}$
SPECIFIC ACTIVITY (percent of injected Ca^{45}/mgm serum Ca)
HOURS
0.10
0.01
0.001
0 9 18 27 36 45 54 63 72 81 90 99
EXPT C
EXPTS.A,B
$C_t = 0.335e^{-0.281t} + 0.205e^{-0.075t} + 0.023e^{-0.004t} + 0.0205e^{-0.0005t}$
SPECIFIC ACTIVITY (% injected Ca^{45}/mgm serum Ca)
0.10 0.08 0.06 0.04 0.02 0.01
0 18 36 54 72 90 108 126 144 162 180
MINUTES

FIG. 1. DISAPPEARANCE OF INJECTED CALCIUM^{45} FROM THE SERUM

Zero time points and zero level points not drawn, but included in derivation of analytical expression which is plotted.

to level off at a constant or equilibrium value.[10] It seemed of interest to derive an analytical expression for the best curve that could be drawn through the experimental points obtained in Experiments A and B. This was done by fitting a weighted sum of exponentials to the data by means of successive isolation.[11] The following expression was obtained:

$$C_t = 0.335e^{-0.281t} + 0.205e^{-0.075t} + 0.0234e^{-0.004t} + 0.0205e^{-0.0005t} \quad (1)$$

where

C_t = per cent of injected Ca^{45} per milligram serum calcium

and

t = minutes after injection

Equation 1 applies to a period of up to four days (6000 minutes) following the injection. Half of the observed experimental points fall within 30 per cent of the values predicted by Equation 1. The curve described by the equation is plotted in Figure 1.

In devising the analytical expression, it was necessary to include calculated zero-time values (total injected Ca^{45}/total serum calcium) in order to anchor down the curve. Inclusion of these values may be criticized because:

(a) Instantaneous mixing of the injected Ca^{45} is assumed, yet mixing is probably not complete for at least 30 seconds (16) and a significant fraction of the injected portion may have disappeared by the time mixing was completed. (See also the curve for Experiment C, Figure 1.)

(b) The lack of data for the time interval 0 to 20 minutes in Experiments A and B makes extrapolation hazardous, as more than one exponential may be involved in that span of time.

If data had been obtained for a longer period than 6000 minutes, it is likely that terms would have to be added to Equation 1 in order to express correctly the events depicted by the experimental observations. On theoretical grounds,

[10] It might be argued that the relatively small quantity of isotope given resulted in erroneously low values as time progressed and that the true values drop off more gradually than shown by the curve in Figure 1. In Figure 3, the very low values were omitted in deriving the group average. As can be seen, there is no indication that the curve reaches an equilibrium value. A similar indication of this is shown in the curves for Experiment C (Figure 4).

[11] Our thanks are due to Dr. J. G. Bryan of the Department of Mathematics, Massachusetts Institute of Technology, for having worked out the method and for having derived the equation for our use.

an expression with no less than five terms is required, if the terms are thought of as describing events in different compartments of the body (at least two compartments in the skeletal systems (17), and one each in the intestinal tract, the serum, and the soft tissues). In the present state of knowledge, however, the terms of Equation 1 ought not to be considered as denoting body compartments, but only as the mathematical representation of experimental observations (10).

Equation 2 can be used to calculate the rate at which the serum calcium leaves the blood (disappearance rate). This rate is defined as the derivative of Equation 1 at time $t = 0$, divided by C_t at time $t = 0$ (*cf.* 18). Calculated for Equation 1 at $t = 0$, the disappearance rate for calcium from the serum is 19.3 per cent per minute.

Minder and Gordonoff (7) obtained disappearance rate curves on rabbits, and a value of 18.2 per cent per minute can be calculated from the expression they reported. However, Thomas, Litovitz, Rubin, and Geschickter (8), who also studied rabbits, reported much higher values, namely 88 ± 36 per cent per minute for old rabbits and 72 ± 12 per cent per minute for young rabbits. The difference between the values for the young and old rabbits was not found to be statistically significant, in contrast to observations on age differences in cattle (10). In the latter species, disappearance rates varied from 102 per cent per min. for calves 10 days old to 26 per cent per min. for cattle 13 years old.

Armstrong, Johnson, Singer, Lienke, and Premer (9), who have conducted similar studies on dogs, report a disappearance rate of 52 per cent per min. with a range of 30 to 60 per cent per minute in six animals.

The experimental limitations involved in determining the early portion of the disappearance curve also limit the interpretations which can be placed on the observed disappearance rate. For this reason, the numerical value reported here should be considered as correct only with respect to order of magnitude.

Figure 1 shows that on a comparable basis a larger fraction of the injected Ca^{45} was detected in the blood of the young adult than in the blood of the boys. Though one individual is not necessarily representative of a group of young adults, the data obtained on this adult subject do not conflict with the observations of Hansard, Comar, and Davis (10) who reported a slower rate of disappearance of injected Ca^{45} in older cattle as compared with young calves.

Urine data

The data in Tables I and II, as well as in Figure 2,[12] appear to suggest that the adolescent boys of Experiments A and B on the whole retained calcium much better than the young adult in Experiment C. However, the output of urinary Ca^{45} by these boys varied from 1.5 to 11.2 per cent of the dose, as shown below:

Subject No.	% Ca^{45} in urine in 5 days	Age (years)
10	1.47	11.2
11	5.91	12.6
12	2.78	12.4
13	6.38	13.1
14	4.82	10.9
15	1.64	15.7
16	6.84	13.1
17	11.20	12.7
18	4.46	11.6
Mean	5.06	12.6
57	13.1	21.0

There was therefore little correlation between chronological age and the urinary calcium output. The extent to which these differences indicate differences in physiological age is problematical.

Figures 3 and 4 indicate the relationship between the specific activity of the urine and the serum. As would be expected, the specific activity values of urine and serum fall on essentially similar curves. Maurer and Zimmer (19) have already described a clinical test where the specific activity of the urine is taken as a measure of the specific activity of the blood.

Blau, Spencer, Swernov, and Laszlo (12) have also reported that the specific activities of serum and urine were equal. However, their data, obtained on two adults, indicate a simple exponential decline with a half-time of eight days. Figures 3 and 4 indicate that no simple exponential decline was observed in our subjects. Furthermore, since the specific activities of urine and serum are alike, Equation 1 with its four terms will also fit the urine data of the boys.

[12] For the purpose of the graphs shown in Figure 2, the values obtained on the 48-hour samples of urine collected in Experiment C were suitably adjusted to represent 24-hour values.

TABLE I

Ca^{40} and Ca^{45} levels in the urine and feces of nine boys following a single intravenous injection of Ca^{45}

Expt.	Subject	Day	Urine			Feces		
			Calcium *mgm.*	Per cent of injected Ca^{45}/mgm. Ca	Per cent of injected Ca^{45}	Calcium *mgm.*	Per cent of injected Ca^{45}/mgm. Ca	Per cent of injected Ca^{45}
B	10	1	44	0.0157	0.69	28	<0.0007*	<0.02*
		2	28	0.0078	0.21	1,064	0†	0†
		3	37	0.0060	0.23	1,082	0.0022	2.40
		4	54	0.0051	0.28	‡		
		5	34	0.0023	0.08	‡		
	11	1	169	0.0210	3.55	1,009	<0.013	<0.1
		2	138	0.0079	1.09	116	0	0
		3	146	0.0041	0.59	662	<0.0012	<0.8
		4	152	0.0035	0.53			
		5	50	0.0030	0.15			
	12	1	64	0.0204	1.30	798	<0.0008	<0.06
		2	66	0.0076	0.50	373	0	0
		3	96	0.0052	0.50	1,306	<0.0002	<0.03
		4	64	0.0038	0.24			
		5	23	0.0102	0.23			
	13	1	167	0.0188	3.12	1,190	0	0
		2	198	0.0074	1.48	822	0	0
		3	266	0.0048	1.28	32	<0.0021	<0.07
		4	83	0.0036	0.30			
		5	121	0.0022	0.20			
A	14	1	151	0.0200	3.02	203	0	0
		2	93	0.0146	1.36	2,011	0.0023	2.24
		3	93	0.0047	0.44	432	0.0050	2.11
		4	sample lost			1,059	<0.0014	<1.5
		5	sample lost			618	<0.0016	<1.0
	15	1	71	0.0156	1.11	604	0	0
		2	62	0.0043	0.27	1,728	<0.0006	<1.0
		3	61	0†	0†	993	<0.0024	<2.3
		4	56	0.0029	0.16	1,074	0	0
		5	80	0.0013	0.10	119	0	0
	16	1	167	0.0216	3.76	901	<0.0003	<0.2
		2	247	0.0059	1.65	1,111	0.0022	2.48
		3	175	0.0033	0.59	1,745	0	0
		4	163	0.0035	0.57	1,463	0	0
		5	126	0.0023	0.27	198	0	0
	17	1	337	0.0204	6.88	—	—	—
		2	279	0.0082	2.31	386	0	<0.02
		3	269	0.0065	1.02	1,802	0.0019	3.44
		4	204	0.0034	0.69	—	—	—
		5	193	0.0029	0.27	930	0	0
	18	1	138	0.0169	2.33	16	0	0
		2	139	0.0064	0.89	1,047	0.0053	1.40
		3	171	0.0042	0.72	509	0.0022	1.11
		4	104	0.0028	0.34	1,550	0	0
		5	116	0.0016	0.18	314	0	0

* The results preceded by the "less than" (<) sign represent counting data which did not differ from background on the 5 per cent significance level, but where all replicate measurements gave counts above background. They are minimum counting values, representing the probable maximum output of Ca^{45} by an individual on that day.

† "0" values represent counting data which did not differ significantly from background.

‡ Because the Ca^{45} content of the feces of subjects No. 10–13 was so very low, analyses were not carried out on the fecal samples of Experiment B which had been collected on Days 4 and 5.

TABLE II

Ca^{40} and Ca^{45} excretion in the urine and feces of a young adult male following a single intravenous injection of Ca^{45} on the morning of Day 1 (Experiment C)

Day	Urine			Feces		
	Ca *mgm.*	Per cent of injected Ca^{45}/mgm. Ca	Per cent of injected Ca^{45}	Ca *mgm.*	Per cent of injected Ca^{45}/mgm. Ca	Per cent of injected Ca^{45}
1	133	0.262	3.49	552	0.00006	0.31
2	262	0.152	4.01	2,510	0.0012	2.90
3	225	0.086	1.94	1,100	0.00067	0.72
4	236	0.089	2.10	853	0.0019	1.64
5	278	0.058	1.60	1,190	0.00097	1.30
8–9	549	0.031	1.69	2,460	0.00042	1.01
15–16	524	0.012	0.60	2,110	0.00017	0.33
22–23	431	0.009	0.37	2,820	0.00013	0.34
29–30	457	0.006	0.25	1,980	0.00008	0.17
57–58	533	0.002	0.12	*	0.00010	0.14

* Repeated analyses gave inconsistent results. Specific activity calculated on basis of each aliquot's actual Ca content.

Feces data

Tables I and II and Figure 5 [13] appear to indicate that the boys in Experiments A and B excreted less Ca^{45} in their feces on the average than the young adult of Experiment C. In view of the reports of Thomas, Litovitz, Rubin, and Geschickter (8) and of Hansard, Comar, and Davis (10), this difference can possibly be interpreted as reflecting higher retention of Ca^{45} by the boys, as compared with the older adult. Only studies with groups of young male adults will make it possible to extend this observation.

The peak Ca^{45} output in the feces occurred on

[13] For the purposes of the graphs shown in Figure 5, the values obtained on the 48-hour samples of feces obtained in Experiment C were suitably adjusted to represent 24-hour values. For this purpose use was made of records kept by the attendant to indicate time and date of defecation on the part of the experimental subject.

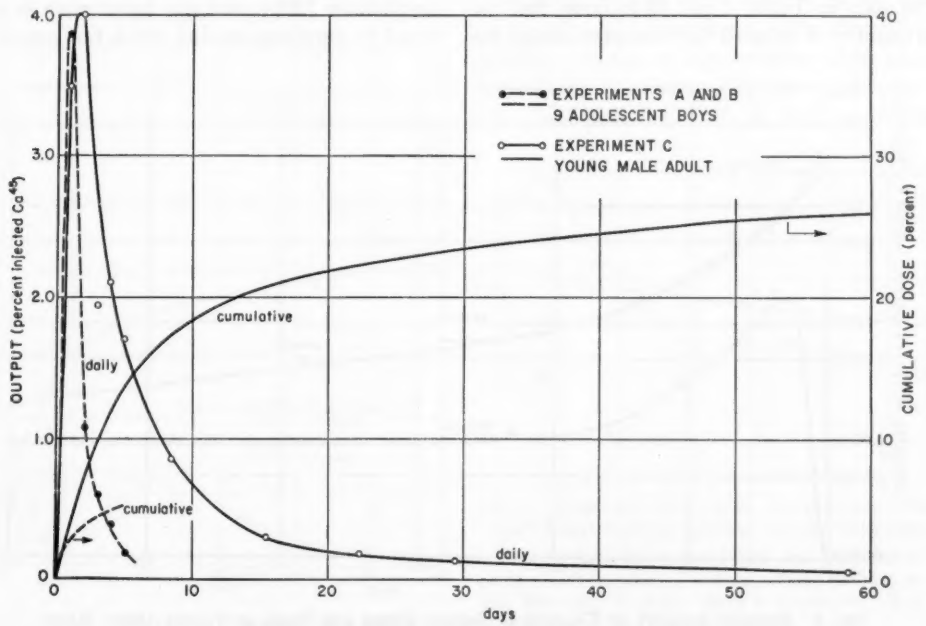

FIG. 2. EXCRETION OF CALCIUM[45] IN THE URINE

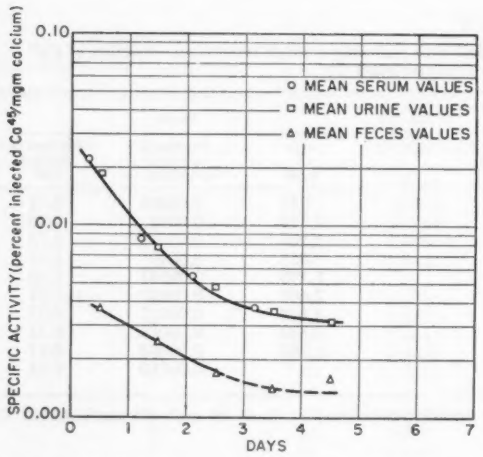

FIG. 3. MEAN SPECIFIC ACTIVITY OF Ca^{45} IN SERUM, URINE AND FECES OF NINE ADOLESCENT BOYS (EXPERIMENTS A AND B)

Counts not differing significantly from background ("0" values, Table I) excluded.

the second or third day, in agreement with the results of Bellin and Laszlo (11).

Combined excretion data

The data in Tables I and II indicate that the total quantity of injected Ca^{45} excreted during the first few days is quite small. In no case did the combined output of isotope exceed 7 per cent of the injected Ca^{45} during any one day (Tables I and II), or 15 per cent during the first five days (Table II). The average combined excretion of the boys in five days was only 8 per cent (Figure 6).

Clearly these observations also apply to the quantity of absorbed Ca^{45} which these subjects might reëxcrete in a comparable period following a test meal containing Ca^{45}. If, for example, one of the boys had eaten two microcuries of Ca^{45} (suitably diluted with ordinary calcium) and if a total of one microcurie had been recovered in the stool at the end of five days, it would be possible to conclude that perhaps $(1 + 0.1)$ microcuries had been absorbed. We have in fact observed similar relationships (1). It may therefore be said that the quantity of calcium which is absorbed and then immediately reëxcreted is quite small; perhaps it may even be neglected in a first approximation because of inherent experimental error (*cf.* [12]).

These considerations suggest that nearly all of the calcium which enters the body at a given moment is at first retained (presumably largely in the skeleton [20]), and that equilibrium is maintained by excreting calcium which had entered the

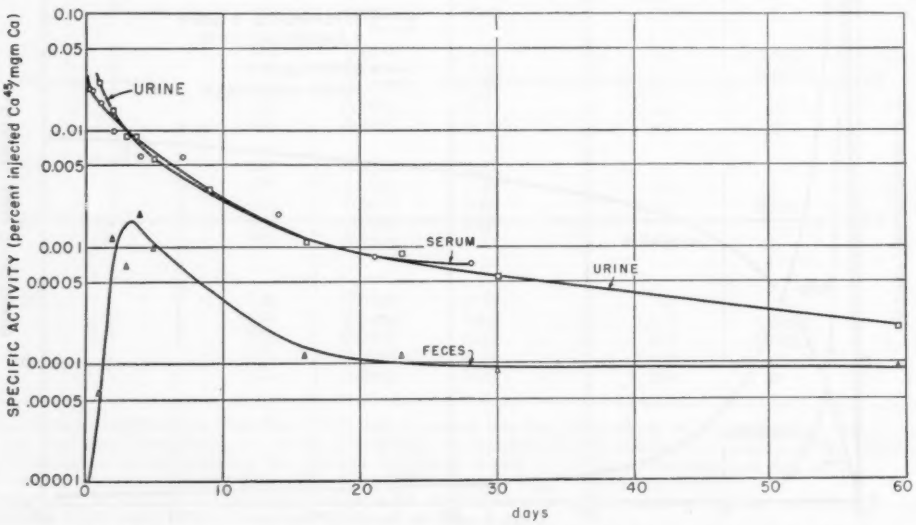

FIG. 4. SPECIFIC ACTIVITY OF CALCIUM IN SERUM, URINE AND FECES OF YOUNG ADULT MALE (EXPERIMENT C)

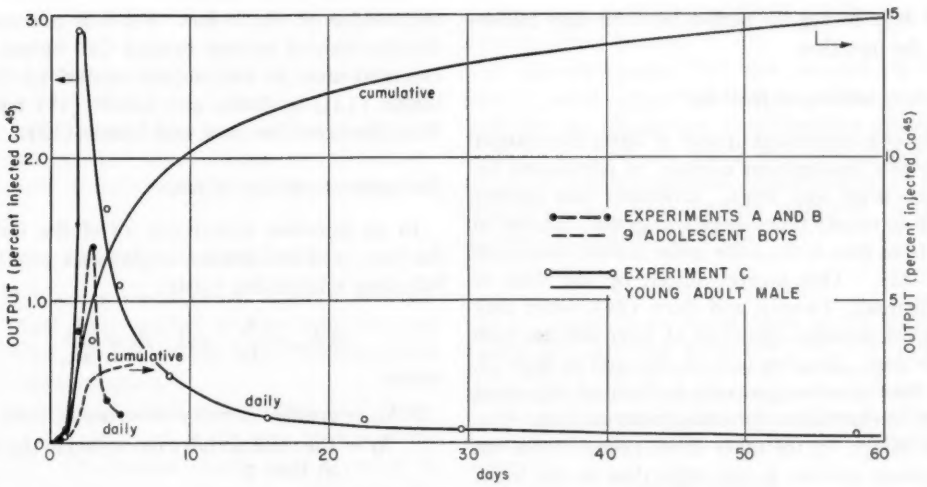

FIG. 5. FECAL OUTPUT OF INJECTED CALCIUM[45]

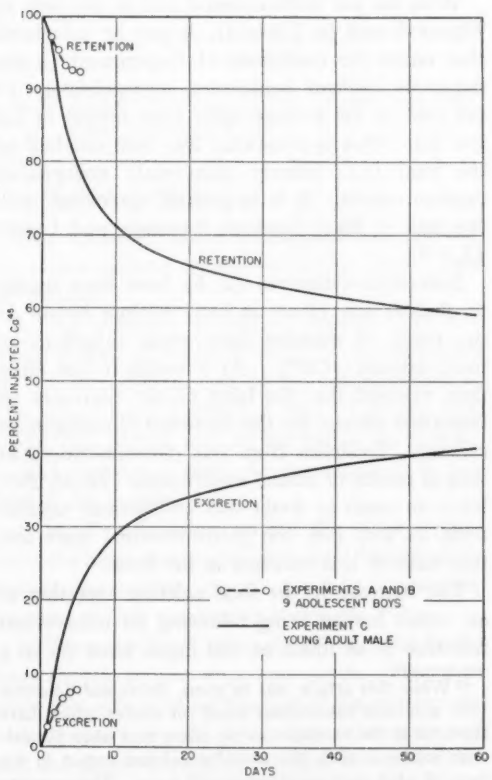

FIG. 6. TOTAL CUMULATIVE EXCRETION AND RETENTION OF INTRAVENOUSLY ADMINISTERED CALCIUM[45]

body previously. This speculation becomes more plausible if the retention of injected Ca^{45} is relatively long. Singer and Armstrong (21) have reported this to be the case for rats.

Figure 6 shows the excretion and retention of Ca^{45} in our subjects. As can be seen, more than half of the injected Ca^{45} was still in the body of subject No. 57 (Experiment C) at the end of two months. A plot of this retention curve on semi-logarithmic coordinates (Figure 7) indicates that the "biological half-life" of Ca^{45} approximates

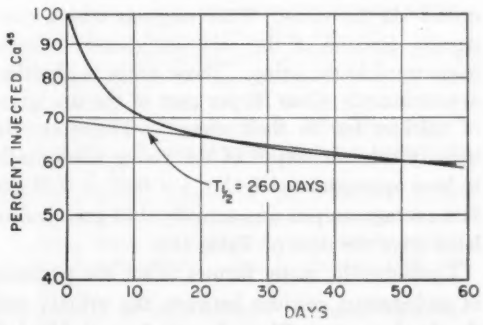

FIG. 7. COMBINED RETENTION (INJECTED Ca^{45}–URINARY Ca^{45}–FECAL Ca^{45}) OF RADIOCALCIUM BY YOUNG ADULT

Best straight line between 18th and 59th day is: $\hat{y} = 69.04 - 0.1784x$, where x = days. Thus, zero day = 69.04 and 59th day = 58.51; $58.51 = 69.04e^{-59a}$, where a = 0.00266; thus, $T_{\frac{1}{2}} = \frac{0.693}{0.00266}$ or 261 days.

260 days during the period 18 to 59 days following the injection.

Ratio of urinary to fecal Ca^{45}

Species differences appear to affect the manner in which "endogenous calcium" is partitioned between urine and feces. Evidently rats excrete eight to twenty times more endogenous calcium in the feces than in the urine under normal conditions (21–23). This is also shown by the data of L'Heureux, Tweedy, and Zorn (24), when they injected minimal quantities of inert calcium with their dose. In cows (10, 25, 26) and in dogs (9, 27) the feces also appears to be the more important route of elimination for endogenous calcium. Human beings, on the other hand, excrete more endogenous calcium in the urine than in the feces. In all of our subjects the urinary fecal ratio of injected Ca^{45} (and therefore also of endogenous calcium) exceeded unity (Tables I and II). This ratio was plotted for subject No. 57, with the cumulative output curves (Figures 2 and 5) as a basis. It remained relatively stable at approximately 1.65 (range: 1.4 to 2.0) during the period of 17 to 59 days following the injection.

The urinary-fecal ratio of injected Ca^{45} can be used to predict urinary calcium output. If it is assumed that all of the injected Ca^{45} will in time be recovered in the urine and feces, then it can be calculated that $\frac{100 \times 1.65}{1 + 1.65}$ or 62.3 per cent of the injected Ca^{45} will eventually have been excreted via the urine. This suggests that a comparable fraction of the absorbed dietary calcium is excreted in the urine. These subjects absorbed approximately 40 to 50 per cent of the one gram of calcium fed in their diets.[14] Therefore, the urinary calcium output of the young adult ought to have approximated $0.45 \times 1 \times 0.62$ or 0.28 gm. His average output was actually 0.24 gm. (calculated from the data of Table II).

Undoubtedly, many factors affect the partition of endogenous calcium between the urinary and the fecal routes. Thus Steggerda and Mitchell (4) were able to increase materially the calcium output in the stool of human subjects by the administration of citrate ion. Yet it is noteworthy that the ratio of urinary to fecal Ca^{45} output also exceeded unity in the subjects studied by Geissberger (13), by Bellin and Laszlo (11) and by Blau, Spencer, Swernov, and Laszlo (12).

Endogenous calcium in feces

In an injection experiment all of the Ca^{45} in the feces is of endogenous origin; as a result, the following relationship exists:

$$S.A._s = \frac{A_t}{E_t} \quad \text{and} \quad E_t = \frac{A_t}{SA_s} \qquad (2)$$

where

$S.A._s$ = specific activity of serum at time t
A_t = the total activity recovered in the feces at time t
E_t = the total quantity of stable endogenous calcium in the feces at time t

With the aid of Equation 2 and of the data in Figure 1 and in Table II, it can be calculated that under the conditions of Experiment C, endogenous calcium constituted approximately 15 per cent of the average daily fecal output of 1.2 gm. Ca. This is somewhat less than one-half of the total (*i.e.*, urinary plus fecal) endogenous calcium output. It is in general agreement with the data of Blau, Spencer, Swernov, and Laszlo (12).[15]

Several investigators (3, 5) have been unable to observe any effect on fecal calcium output as the result of massive intravenous injections of inert calcium (Ca^{40}). As a result it has often been asserted that the feces do not represent an important avenue for the excretion of endogenous calcium. Evidence from this communication, as well as results of animal experiments (22–26, 29), leave no room to doubt that endogenous calcium finds its way into the gastrointestinal tract and that some of it is excreted in the feces.

The increase in the fecal calcium excretion of an adult human being following an intravenous injection of as much as 500 mgm. inert Ca in a

[14] On the basis of studies to be published. Similar relationships are also reported by Blau, Spencer, Swernov, and Laszlo (12).

[15] While this article was in press, Brine and Johnston (28) published calculations based on studies which have appeared in the literature to the effect that when the calcium intake is zero, the probable calcium output in the feces of adult man approximates 75 mgm. This quantity may be considered the endogenous calcium output and is reasonable confirmation of the studies reported here.

day would probably amount to no more than 50 mgm., or five to ten per cent of his daily fecal output (on the assumption that the calcium is injected under physiological conditions). Clearly this increase would be difficult to detect and experiments in which inert calcium is injected intravenously therefore tend to lead to negative results.

SUMMARY AND CONCLUSIONS

1. The fate of intravenously injected radiocalcium (Ca^{45}) was studied in nine boys and one young adult, all of whom had been institutionalized for mental inadequacy.

2. The rate at which the injected Ca^{45} disappeared from the blood of the nine boys was determined. Observations made over a period of five days, together with assumed zero-time values, were expressed by the equation:

$$C_t = 0.335e^{-0.281t} + 0.205e^{-0.075t} + 0.0234e^{-0.004t} + 0.0205e^{-0.0005t} \quad (1)$$

where

C_t = specific activity of the serum (per cent dose per mgm. Ca)

and

t = time (minutes)

This equation yielded a disappearance rate for the serum calcium of approximately 19 per cent per minute.

3. The average quantity of injected Ca^{45} excreted in the urine and the feces by the nine boys did not exceed 8 per cent by the end of five days. The young adult excreted a total of 20 per cent during this period, and, by the end of sixty days, he had excreted a total of approximately 40 per cent of the injected dose.

4. In all individuals studied the urine constituted the more important route of Ca^{45} elimination and, on the average, contained 1.5 to 2 times the quantity of Ca^{45} excreted in the feces.

5. The quantity of injected Ca^{45} excreted in the feces during several days was small. This suggests that only a minor fraction of the calcium absorbed on any one day is reëxcreted promptly and that the major portion is retained for some time. The "biological half-life" of Ca^{45} in one young adult was calculated to approximate 260 days, during the period of 18 to 59 days following the injection of the isotope.

6. Because injected Ca^{45} was recovered in the feces it would appear that the feces serve as one route for the elimination of endogenous calcium. Data obtained on one young adult over a period of two months indicate that approximately 15 per cent of his average daily fecal output of 1.2 gm. Ca was endogenous in origin.

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THE ANEMIA OF INFECTION. XX. THE KINETICS OF IRON METABOLISM IN THE ANEMIA ASSOCIATED WITH CHRONIC INFECTION[1]

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Although the morphologic, biochemical and clinical characteristics of the anemia associated with chronic infection have been studied extensively (1), the pathogenesis of the anemia remains obscure. The profound alterations in iron and porphyrin metabolism which accompany this type of anemia have been interpreted as indicating a decreased rate of erythrocyte production as the result of a quantitative defect in the rate of conversion of protoporphyrin to hemoglobin (1, 2). The usual evidences of increased blood destruction, such as increased urobilinogen excretion and elevated serum bilirubin concentration, are lacking in the anemia of chronic infection (1).

Studies of the erythrocyte survival time have yielded conflicting results. Mollison (3), using the Ashby differential agglutination technique, found a normal disappearance rate for erythrocytes transfused into six patients with chronic sepsis and anemia. Brown (4), employing the same technique, observed a decreased life span of transfused erythrocytes in four of five cases. In pulmonary tuberculosis Hollingsworth and Hollingsworth (5) reported recently that the life span of autotransfused Cr^{51}-tagged erythrocytes is normal.

Since the anemia which is associated with rheumatic fever and rheumatoid arthritis is morphologically and biochemically similar to that associated with chronic sepsis (1), it seems appropriate to comment on several studies in these conditions. Reinhold (6), using the Ashby method, found the erythrocyte life span to be decreased in children with acute rheumatic fever. By the use of the Fe^{59} tracer technique and the Ashby method, Freireich, Ross, Bayles, Emerson, and Finch (7) found the erythrocyte life span to be decreased in rheumatoid arthritis. These investigators presented evidence suggesting that the shortened erythrocyte life span was due to an extracorpuscular hemolytic mechanism. Ebaugh, Peterson, and Bunim (8), employing the Ashby technique, the autotransfusion of Cr^{51}-tagged erythrocytes, and the Fe^{59} tracer technique, also found the erythrocyte life span to be shortened in rheumatoid arthritis.

Huff, Hennessy, Austin, Garcia, Roberts, and Lawrence (9) have developed a method for the determination of the turnover rate of iron through the plasma and red cells. By the use of these rates it is possible to understand better the alterations in the kinetics of iron metabolism which occur in various anemias (10–22). In addition, an estimation of the life span of the red cells can be obtained. The purpose of this report is to present the results of such studies on six patients with the anemia of chronic infection and, for comparison, on ten normal subjects. A preliminary report of this work has been published (23).

MATERIAL AND METHODS

The clinical and hematologic data are summarized in Table I. The normal subjects were healthy young males. All of the patients were males with no evidence of diseases other than those listed. During the period of study each subject was in a steady state with respect to erythrocyte production and destruction since no significant changes in the volume of packed red cells were noted. The reticulocyte values represent the means of at least 10 counts on each individual. All determinations were performed by the same technician. Although the individual mean values were all within normal limits for this laboratory, the difference between the mean value obtained on the normal subjects and that

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TABLE I

Clinical and laboratory findings on normal subjects and patients

Patient	Age (*years*)	Diagnosis	Duration of infection (*weeks*)	V.P.R.C.* *ml./100 ml.*	Body temperature (average daily maximum)	White count *cells/cu. mm.*	R.B.C. vol. *ml./Kg.*	MCHC % †	Plasma iron *µg./100 ml.*	Reticulocytes %	Serum bilirubin *mg./100 ml.*	Hemolytic index
Normal subjects		Mean		48			36.3	34	125	0.8		
		Range		46–51			30.7–43.8	32–35	94–150	0.4–1.3		10–24
A. M.	64	Silicotuberculosis with secondary aspergillosis	48	36	99.6	7,500	34.9	32	34	1.1		18
F. B.	65	Tuberculosis (osseous and pulmonary)	18	33	100.5	8,200	27.0	32	30	1.7		3
W. S.	46	Chronic lymphangitis and cellulitis, right leg	14	43	99.0	9,800	32.8	33	57	1.5	0.8	
W. F.	60	Unresolved pneumonia	3	35	100.0	13,500	36.0	33	30	1.6	0.4	11
P. S.	64	Severe tuberculosis (pulmonary)	14	36	100.0	14,400	26.9	32	39	1.1	0.8	
R. D.	33	Tuberculous pneumonia	10	39	102.0	16,900	25.7	34	38	0.9	0.4	15
Mean				37			30.6	33	38	1.3		

* Volume of packed red cells.
† Mean corpuscular hemoglobin concentration.

obtained on the patients with infection is significant ($t = 7.35$, $P < .01$).

Plasma iron determinations were performed by the method of Hamilton, Gubler, Cartwright, and Wintrobe (24). Fecal urobilinogen was determined by the method of Schwartz, Sborov, and Watson (25). The hemolytic indices were calculated by the method of Miller, Singer, and Dameshek (26). The volume of packed red cells, hemoglobin concentration, and reticulocyte per cent were determined daily.

The radioactive iron was supplied as ferric (Fe^{59}) chloride on allocation from the U. S. Atomic Energy Commission. Ferric chloride, containing 3 to 6 µg. of iron and 5 to 20 µc of radioactivity, was buffered to pH 6.0 with 4 per cent sodium citrate and incubated for 30 minutes at room temperature with 12 ml. of the subject's own plasma. Ten ml. of this material were injected intravenously at 9:00 A.M. with the subject in the post-prandial state. Plasma samples were obtained at 15 to 30-minute intervals following the injection until the activity in 1 ml. of plasma had decreased to twice the background count. The samples were counted for 4,096 counts in a well-type, thallium-activated sodium iodide crystal, scintillation counter. A standard was prepared by diluting 1 ml. of the remaining plasma-Fe^{59} mixture to 500 ml. with water. One ml. of this material was pipetted and retained as a reference standard for subsequent counts.

The plasma iron turnover rate in ml. per Kg. of body weight per day was determined by the method of Huff and his co-workers (9), by the use of the following formula:

$$\frac{.693 \times \text{Plasma iron (mg./ml.)}}{T\ 1/2\ \text{(hours)} \times \text{body weight (Kg.)}} \times \text{Plasma volume (ml.)} \times 24\ \text{(hours)} = \text{Plasma iron turnover rate}$$

The number 0.693 is the natural log of 2.

The plasma iron value taken for the calculations was the mean of the values of all samples from time zero to T ½.

The T ½ is the time at which the activity in the plasma had decreased to half of the initial concentration. For this determination the counts for the individual plasma samples were plotted against time on semi-logarithmic paper. A line was fitted to the points by eye and extrapolated to time zero.

The plasma volume was determined on the last day of the study with 5 µc of I^{131} human serum albumin (27). A few ml. of normal human plasma were added to the standard in order to eliminate attraction of the protein to the walls of the glassware (28).

Samples of blood were obtained daily at 9:00 A.M. until the activity in the red cells failed to increase for three successive days. For counting, 1 ml. of blood was hemolyzed with saponin; whole blood, rather than washed red cells, was counted because no significant radioactivity was present in the plasma after the day of injection. In order to correct for radioactive decay, the standard was counted daily.

The total blood volume was determined by appropriate calculations from the plasma volume and the volume of packed red cells. The total amount of radioactivity present in the red cells was determined by multiplying the counts in 1 ml. of blood by the blood volume. The frac-

tion of injected Fe^{59} incorporated into the red cells was determined by dividing the total amount of radioactivity found in the red cells by the activity injected (counts per minute per ml. of standard × 500 (dilution of standard) × 10 (ml. of plasma injected)).

The red cell iron turnover rate in mg. per Kg. per day was determined by the following formula (9):

Plasma iron turnover rate × maximum fraction of Fe^{59} incorporated into red cells = Red cell iron turnover rate, where:

The maximum fraction of Fe^{59} incorporated into red cells is the mean of the values obtained on the three consecutive days in which the proportion of radioactivity in the erythrocytes was no longer increasing.

The total amount of red cell iron, expressed in mg., was calculated by the use of the following formula:

$$\frac{\text{Blood volume (ml.)} \times \text{Hemoglobin (gm./100 ml.)} \times 3.4}{100} = \text{Red cell iron}$$

The fraction of red cell iron renewed each day was determined by the following formula (9):

$$\frac{\text{Red cell iron turnover rate} \times \text{Body weight in Kg.}}{\text{Red cell iron}} = \text{Fraction of red cell iron renewed per day}$$

The reciprocal of the fraction of red cell iron renewed each day is the red cell life span.

Body-surface counting was performed by the use of a mobile scintillation counter (10). The skin was marked with ink at appropriate points over the liver, spleen, sacrum, and rib cage. Since the liver and spleen were not palpable in any of the subjects, the sites for counting these organs were determined by percussion, and the counter was directed at the organ through an intercostal space. In order to eliminate the influence of activity emanating from costal bone marrow, an intercostal space in the right axilla was counted also and this value was subtracted from that obtained at the same time over the liver and spleen, respectively.

The counts obtained at each site were corrected for activity due to blood circulating through the area in the following manner (13): Immediately after injection of the labelled plasma, serial counts were obtained at each site. By plotting these counts on semi-logarithmic paper and extrapolating the line to time zero, the activity present at a particular site when 100 per cent of the injected activity was in the blood was determined. At the time of each *in vivo* count, the counts obtained were corrected for activity due to blood by the use of the following formula (10):

$$CPM^{s} - CPM^{o} \times \frac{Bl^{act}}{100} = CPM^{s}$$

where:

CPM^{s} is the gross counts per minute obtained at any counting site;

CPM^{o} is the activity present in the counting site when 100 per cent of the injected activity was in the blood;

Bl^{act} is the per cent of injected activity present in the blood (plasma and/or red cells) at the time of the particular counting;

CPM^{s} is the corrected counts per minute.

RESULTS

Turnover of Fe^{59} through plasma

The injected Fe^{59} disappeared from the plasma at a single exponential rate in all 6 patients with the anemia of infection and in 8 of the 10 normal subjects. Two of the normal subjects exhibited a slightly more rapid phase of isotope clearance during the first hour. However, during the next four hours the activity in the plasma diminished at a single exponential rate. The second rate was considered to represent the true rate of Fe^{59} disappearance in these 2 cases (21).

The curves of disappearance of Fe^{59} from the plasma of the normal subjects and of the patients

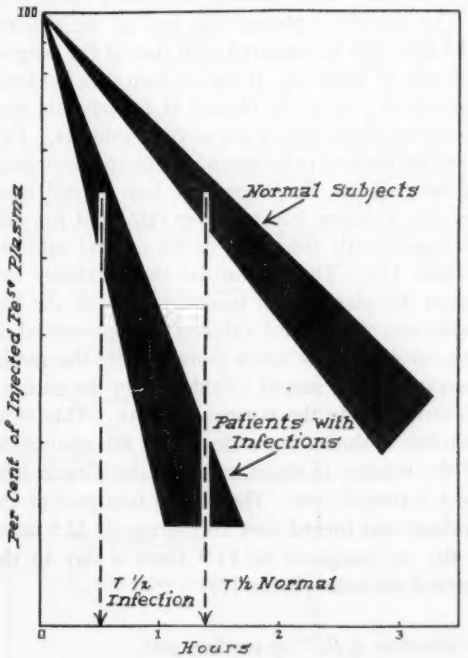

FIG. 1. DISAPPEARANCE OF Fe^{59} FROM PLASMA OF PATIENTS WITH ANEMIA OF INFECTION COMPARED TO DISAPPEARANCE FROM PLASMA OF NORMAL SUBJECTS (RANGE OF VALUES)

T ½ is the time at which the concentration of Fe^{59} in the plasma had decreased to half of its initial value.

TABLE II

Plasma iron turnover data on normal subjects and patients

Patient		Half time of plasma Fe^{59} disappearance (*Hours*)	Plasma iron pool (*mg.*)	Plasma iron turnover rate *mg./Kg./day*	No. of times/day plasma iron pool turned over
Normal subjects	Mean	1.39	3.5	0.56	11.9
	Range	1.05–1.73	2.36–4.63	0.46–0.75	9.6–15.9
A. M.		0.57	0.9	0.65	29.3
F. B.		0.42	0.8	0.65	39.6
W. S.		0.62	1.9	0.65	26.8
W. F.		0.47	1.0	0.58	35.3
P. S.		0.62	1.1	0.54	27.0
R. D.		0.38	0.9	0.59	36.7
Mean		0.51	1.1	0.61	32.5

TABLE III

Red cell iron turnover data on normal subjects and patients

Patient		Mean maximal Fe^{59} uptake in red cells	Red cell iron turnover rate *mg./Kg./day*	Red cell iron renewed daily %	Red cell life span (*days*)
Normal subjects	Mean	95	0.52	1.28	79
	Range	83–100	0.43–0.72	0.97–1.45	69–103
A. M.		96	0.62	1.60	62
F. B.		98	0.64	2.15	47
W. S.		100	0.65	1.78	56
W. F.		92	0.53	1.52	66
P. S.		95	0.51	1.61	62
R. D.		87	0.51	1.75	57
Mean		95	0.58	1.73	58

with chronic infections are shown in Figure 1. The average time at which half of the activity initially present had disappeared (T ½) in the patients with infection was 0.51 hours as compared to 1.39 hours in the normal subjects (Table II).

The size of the plasma iron pool of the patients with infection is compared with that of the normal subjects in Table II. It can be seen that the total amount of iron in the plasma of the patients was about one-third that of the normal subjects. Despite the marked reduction of the plasma iron pool in the patients, the amount of iron turned over per day (plasma iron turnover rate) did not differ significantly from that of the control subjects (Table II). The reason for the similarity between the plasma iron turnover rates of the patients and the control subjects is apparent when one considers how much more rapidly the radioiron tracer was moved (Table II) in the patients as compared to the normal subjects. This relationship is shown more clearly by the calculation of the number of times each day the plasma iron pool is turned over. The plasma iron pool of the patients was turned over an average of 32.5 times a day as compared to 11.9 times a day in the normal subjects (Table II).

Utilization of Fe^{59} by erythrocytes

The uptake of Fe^{59} by the erythrocytes of the patients with chronic infection is compared to that of the normal subjects in Figure 2. The average proportion of injected Fe^{59} in the erythrocytes of the patients was higher on the 1st, 2nd, and 3rd days after injection than in the normal subjects. Maximal incorporation was attained in the normal subjects between the seventh and the tenth day, and in the patients between the fifth and eighth day after the injection. At the time of the maximal incorporation of Fe^{59} into the red cells, the patients had incorporated an average of 95 per

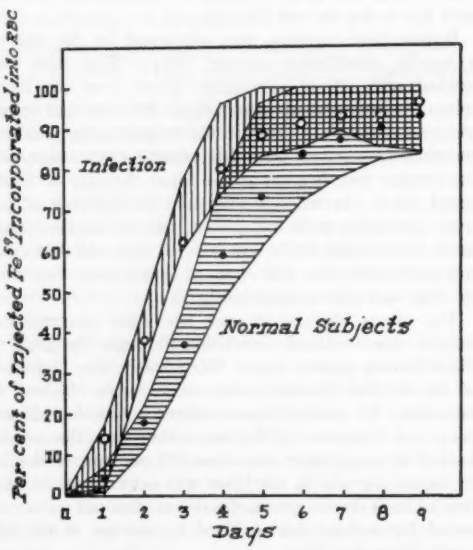

FIG. 2. UPTAKE OF Fe^{59} INTO ERYTHROCYTES OF PATIENTS WITH ANEMIA OF INFECTION COMPARED WITH NORMAL SUBJECTS

The vertical lines represent the range of values in the patients with infections. The horizontal lines represent the range of values in the normal subjects. The circles and dots represent the mean values in the patients and normal subjects, respectively.

cent of the isotope. This was the same as the value obtained in the normal subjects (Table III).

The mean red cell iron turnover rate in the patients was 0.58 mg. per Kg. per day as compared to 0.52 mg. per Kg. per day in the control subjects (Table III). The average per cent of red cell iron renewed each day in the patients with infection was 1.73 as compared with 1.28 in the normal subjects (Table III), and the average red cell life span was 58 days as compared to 79 days in the normal subjects.

Body-surface counts

Graphs of the body-surface counts in two normal subjects (L. L. and J. J.) and in two patients with infection (R. D. and A. M.) are shown in Figure 3. The pattern did not differ significantly between the two groups. The activity at the bone marrow site (sacrum) increased rapidly and peak activity was attained between 4 and 6 hours after the time of injection. Thereafter the activity decreased at a rate that was roughly the inverse of the curve of uptake of Fe^{59} into the red cells (Figure 2). A small proportion of activity entered the spleen and liver in one of the normal subjects (J. J.) and one of the patients with infection (R. D.). In the other two subjects no significant activity was detected in either the liver or the spleen.

DISCUSSION

The data presented indicate that the amount of iron turned over through red cells each day by the patients with infections was approximately the same as that turned over by normal subjects. Expressed in another way, the patients with infection made and destroyed each day the same amount of hemoglobin as did the normal subjects. Since the patients were anemic and the total red cell iron was reduced, the fraction of red cells renewed each day $\left(\frac{\text{red cell iron turnover rate}}{\text{total red cell iron}}\right)$ was increased and, hence, the erythrocyte life span was shortened.

At first glance, it would seem that the anemia is explained by the shortened life span of the erythrocytes. However, the reduction in red cell longevity is slight when compared to that observed in hemolytic anemia (29). In our patients with infections a 50 per cent increase in erythropoiesis would have been sufficient to prevent the occurrence of anemia. When consideration is given to the observation that the normal bone marrow is capable of increasing erythrocyte production 6 to 8-fold (29), it is apparent that there is marked inhibition of the bone marrow in patients with infection. Furthermore, although anemia would not develop in the absence of a decrease in red cell life span, the inability of the bone

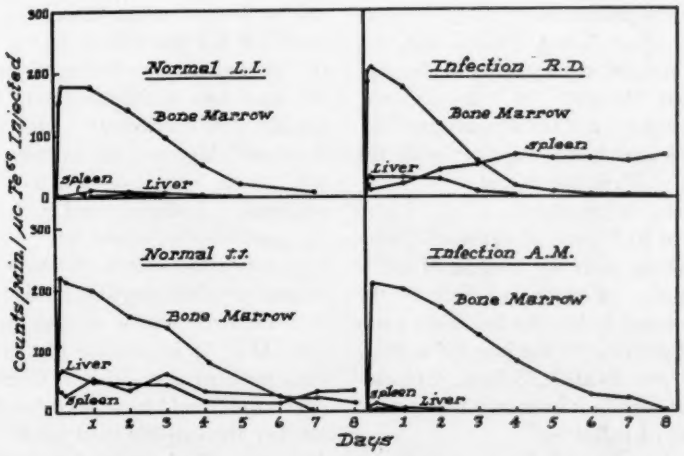

FIG. 3. BODY-SURFACE COUNTS OBTAINED OVER LIVER, SPLEEN, AND SACRAL BONE MARROW OF TWO PATIENTS WITH ANEMIA OF INFECTION (R. D. AND A. M.) AND TWO NORMAL SUBJECTS (L. L. AND J. J.)

marrow to increase erythroycte production is a defect of greater magnitude than the decrease in red cell longevity.

It should be pointed out that all six of the patients in this study had chronic infections and a mild degree of anemia which was neither improving nor becoming more severe. Therefore, these observations pertain only to patients of this type and it cannot be inferred that all patients with infection will have a similar ferrokinetic pattern.

The patients in this study incorporated an average of 95 per cent of the injected activity into the erythrocytes. Furthermore, the rate of incorporation was more rapid than in the normal subjects. These observations are in contrast to those of earlier investigators (30, 31) including studies in our own laboratory (32). In these investigations it was found that both the rate and the amount of radioiron incorporated into the red cells were decreased in patients with chronic sepsis. Furthermore, the extent of this decrease was dependent on the severity of the infection. A reasonable explanation to account for this difference is that the earlier investigators administered considerably larger quantities of carrier iron. For example, Dubach, Moore, and Minnich (30) administered 2.5 to 22 mg. of iron; Finch Gibson, Peacock, and Fluharty (31) gave 0.1 to 0.5 mg. and Greenberg, Ashenbrucker, Lauritsen, and Wintrobe (32), 3 to 4 mg. The amount of carrier iron used in the present study was between 3 and 6 μg. Since the plasma iron pool in patients with infection is about 1 mg. (Table II), the amounts of iron injected in the earlier studies appreciably increased this pool. As a consequence, it is probable that a portion of the administered isotope was shunted into stores together with the plasma iron with which it had mixed.

In order to test this hypothesis, 3 mg. of iron containing 10 μc of Fe^{59} were administered intravenously to a patient with the anemia of infection (33). The rate of uptake of Fe^{59} by the erythrocytes was found to be retarded and a maximum of only 60 per cent of the injected activity was found in the red cells after 12 days. Unfortunately it was not possible to restudy this patient with a tracer dose of radioiron.

By the use of a variety of direct methods for determining red cell life span, it has been shown that the normal human erythrocyte persists in the circulation for 110 to 120 days (34). The red cell life span as calculated from the ferrokinetic data was 79 ± 11 days in our normal subjects. This is in close agreement with the values for life span calculated from ferrokinetic data reported by some workers (20, 21), although it is somewhat shorter than the mean life span calculated from the data reported by Huff and his associates (9). There are several possible explanations for these low values for erythrocyte life span in normal subjects. First, the blood volume determinations may be erroneously high. The calculation of total blood volume on the basis of plasma volume determinations has been shown to give values which are too high, especially when the subjects are not anemic (35). On the other hand, when the total blood volume is calculated by an erythrocyte-tagging method, the value is too low (35). The true blood volume is probably obtained by the summation of the plasma volume and the red cell mass, determined simultaneously by appropriate specific methods. However, the validity of this assumption does not lend itself readily to experimental confirmation. Because of the simplicity and reproducibility of the determination of plasma volume with I^{131} human serum albumin, this method was employed with a full realization of its limitations. Furthermore, no correction was made for the difference between body and venous hematocrit because it has been our experience that this ratio is not constant from one individual to another. No correction was made for trapped plasma in the hematocrit since the correction curve had not been determined for the conditions of this laboratory at the time these studies were carried out. Had these corrections been used, however, the calculated blood volumes would have been lower and as a consequence the percentage of administered Fe^{59} incorporated into the erythrocytes would have been less and the erythrocyte life span would have been longer. A second possible explanation is that the plasma iron turnover rate is subject to a diurnal cycle (36, 37). It is possible that if the studies had been initiated at a different time of day the turnover rates would have been lower and the calculated erythrocyte life span would have been longer. However, all of the studies were initiated at the same time of day. Another possibility is that a portion of the iron which is ultimately incor-

porated into hemoglobin does not enter the bone marrow directly from the plasma but may be cycled first through other channels. If such were the case the calculated values for the turnover rates would be falsely high and the red cell life span would be falsely low. Certainly additional unknown errors may be present in the ferrokinetic studies and for the present the values for life span must be considered as relative and not absolute values. However, it is likely that the observed differences between the two groups are real since similar methods were used and similar errors were involved.

The pattern of movement of Fe^{59}, as determined by body-surface counting, was not different in the patients with the anemia of infection as compared with that in the normal subjects. The appearance of almost all of the radioactivity in the bone marrow between 4 and 6 hours after injection is in keeping with the high proportion of the administered radioactivity which was ultimately incorporated into hemoglobin. It is interesting that the patterns observed in both the normal subjects and the patients in this study are in marked contrast to those observed by Huff and his co-workers (10). These investigators noted considerable activity in the liver and spleen during the first five days of the study. However, although it was necessary for them to direct their counter at the liver and spleen through intercostal spaces, these workers did not make a correction for activity originating in the bone marrow of the ribs. When our data are plotted without the correction for activity originating in rib marrow, the patterns are similar to those described by Huff, and his co-workers. In view of the considerable quantity of active marrow which is present in the ribs, it seemed appropriate to correct the counts obtained over the liver and spleen sites. Furthermore, since in our subjects an average of 95 per cent of the administered activity was incorporated into hemoglobin, it would be surprising if a significant proportion of the radioactivity entered organs other than the bone marrow.

SUMMARY

1. Plasma and red cell iron turnover studies were carried out on 6 patients with the anemia of infection and on 10 normal subjects. Body-surface counts were made with a mobile scintillation counter over the liver, spleen, and sacral bone marrow sites in two patients and in two control subjects.

2. The average half time of plasma Fe^{59} disappearance in the patients was 0.51 hours as compared with 1.39 hours in the control subjects. The average plasma iron turnover rate and red cell iron turnover rate in the patients were 0.61 mg. per Kg. per day and 0.58 mg. per Kg. per day, respectively, as compared with 0.56 mg. per Kg. per day and 0.52 mg. per Kg. per day in the normal subjects. The incorporation of Fe^{59} into the red cells of the patients was initially more rapid than in the control subjects. The ultimate percentage of injected Fe^{59} incorporated into erythrocytes was the same in both groups. The average "apparent" erythrocyte life span as calculated from the above data, was significantly shorter in the patients than in the control subjects. The body-surface counting patterns of uptake and delivery of Fe^{59} by the liver, spleen, and bone marrow in the patients with infection did not differ significantly from those in the normal subjects. Very little activity entered the liver or spleen.

3. These data suggest that the erythrocyte life span in patients with chronic infection is shortened. However, the bone marrow is unable to compensate for this modest shortening of the erythrocyte life span. When consideration is given to the fact that a normal marrow is capable of increasing its production 6 to 8-fold, it is apparent that there is marked inhibition of erythropoiesis in such patients.

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THE RESPONSE OF BONE, CONNECTIVE TISSUE, AND MUSCLE TO ACUTE ACIDOSIS [1, 2]

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Previous evidence has indicated that under the influence of diverse stimuli, variable quantities of sodium, chloride, and water may be rapidly transferred into the fluid phase of the extracellular compartment (1–3). The precise source of these electrolytes has not been established. Since the tracer substances used to measure the fluid phase of the extracellular compartment (by the dilution technique) are excluded from the relatively solid phases of this compartment (bone and connective tissue), from the gastrointestinal tract and from cells (4–6), any one or combination of these sites could serve as a source of these electrolytes.

The possibility that the solid phase of the extracellular compartment may serve as an immediate reservoir of electrolytes is particularly intriguing since these solid phase tissues are characterized by excesses of specific electrolytes; sodium in the case of bone and presumably chloride in connective tissue (7, 8). It has recently been demonstrated that after two days of a combined stimulus of sodium depletion, chloride administration, potassium depletion and starvation, considerable quantities of sodium and potassium may be mobilized from rat bone (9, 10). It was not determined whether these electrolytes derived from cellular bone marrow or acellular bone cortex or possibly both sites. Furthermore that bone cortex *per se* may rapidly release considerable quantities of confined sodium and connective tissue release or alter the excess electrolytes contained therein to defend against an acute alteration in extracellular fluid composition has similarly not been established.

Accordingly, direct water and electrolyte analyses of marrow-free cortical bone and dense connective tissue (tendon) from the rat were performed to determine first, the quantities of electrolyte contained therein not attributable to extracellular fluid and second, whether these excesses might be rapidly mobilized by acute extracellular acidosis. Muscle and red cells were similarly analyzed to compare the response of such relatively cellular tissues with that of acellular solid phase extracellular tissues. The experiments were terminated four hours after the administration of a large ammonium chloride or hydrochloric acid load in order to define the extent to which endogenous redistribution of specific electrolytes preserved extracellular fluid composition prior to effective renal compensation.

METHODS

Normal male albino rats weighing 250 to 350 grams and maintained on a regular diet were injected intraperitoneally with one of two acid solutions. In the first group a solution of 100 mEq. per L. ammonium chloride was administered in amounts of 12 cc. per 100 grams of rat; in group number II, a 250 mEq. per L. solution of ammonium chloride or hydrochloric acid was injected in doses of 5 cc. per 100 grams of rat. The total chloride load administered was approximately equivalent in both groups but in group I, the administered solution was hypotonic whereas in group II a hypertonic solution was used. After the injection, the animals were placed in individual metabolism cages, fasted, and all urine formed was collected. Four hours after injection, the animals were lightly anesthetized with ether and immediately sacrificed by rapid exsanguination from the heart. Uninjected rats of similar size, maintained on the same diet and fasted for a comparable period, were sacrificed in the same way to serve as a control group. Plasma sodium, chloride, potassium and carbon dioxide concentrations, plasma pH and whole blood hematocrits were measured in the normal and acidotic rats. The volume of urine and residual peritoneal fluid as well as their respective electrolyte concentrations were also measured

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in the acidotic rats. Bone cortex, tendon, and muscle were analyzed for water, sodium, chloride, potassium, and calcium (in the case of bone) content in all rats.

Fragments of long bones were dissected from the lower extremities, and meticulously cleaned of adhering muscle and fat. *All marrow was removed.* Tendon was obtained from the tail and dissected free of adhering muscle or loose connective tissue. This latter procedure was performed in a cold, humid atmosphere to minimize loss of water from evaporation. Muscle was obtained from the lower extremities and quickly cleaned of visible fat and connective tissue. The tissues thus prepared were placed immediately in weighing bottles, minced, weighed, and the water content determined by drying to constant weight at 105°C. The bone was ground to a very fine powder after a preliminary 24-hour period of drying and then dried further to constant weight.

The sodium and potassium content of bone, tendon, and muscle were determined by the wet digestion of dried finely ground aliquots of tissue in a 1:1 mixture of concentrated sulfuric acid and perchloric acid, subsequent dilution and analysis in a Baird Associates internal standard Flame Photometer. Calcium analyses of bone were performed by ashing a finely ground sample in a muffle furnace at 500°C. for at least 48 hours and by subsequent precipitation of the calcium as calcium oxalate, according to the method of Bergstrom and Wallace (9). It has been previously contended that by the method of flame photometry, the presence of quantities of calcium found in bone may interfere with the sodium readings (11). Accordingly, known solutions of calcium and sodium containing quantities of these ions to be expected in these bone samples were subjected to this wet ashing technique and then analyzed for sodium content. No detectable influence on the sodium reading was apparent. Furthermore, the washed calcium-free supernatant from the dry ashed bone was analyzed for sodium and the results obtained from this dry ashing technique agreed with the sodium content determined by the wet ashing technique within 4 per cent.[3] Accordingly, the sodium analyses were performed by the simpler wet ashing technique.

The chloride content of tendon and bone was measured by the nitric acid digestion technique as modified by Sunderman and Williams (12). Muscle chloride analyses were performed by the nitric acid extraction technique of Hastings and Eichelberger (13). Plasma, peritoneal fluid, urinary sodium and potassium concentrations were measured by flame photometry, similar chloride concentrations by the Volhard technique (14). Plasma pH measurements were made on a Beckman pH meter with proper correction for room temperature. Carbon dioxide content was measured by the method of Van Slyke and Neill (15) and hematocrit by the standard Wintrobe tube. Insofar as relative changes after an acute stimulus were under study and as the animals were sacrificed by exsanguination, no corrections were made for fat content and blood contamination of muscle. The fat content and blood contamination of bone cortex and tendon of the rat are not significant.

Any conclusions regarding changes in the extracellular fluid of specific tissues studied are based upon the hypothesis that interstitial fluid or the fluid phase of the extracellular compartment bears an electrolyte composition identical to that of a serum ultrafiltrate. In this view, if all the sodium and chloride content of an organ is attributable to such fluid, the ratios of these ions should equal the ratios in serum ultrafiltrate. If one electrolyte is present in excess of the ratio found in serum, at least that amount in excess cannot be attributed to interstitial fluid. Under this same basic assumption, the volume of distribution of either sodium or chloride in any tissue, which ever be smaller, represents the maximal volume of interstitial fluid which that organ may contain.

RESULTS

The data on electrolyte and water content of the tissues from the normal rats are presented in Table I. Bone cortex contained approximately 0.29 mEq. of sodium per gm. of dry tissue, about 1/9th as much chloride or 0.03 mEq. of chloride per gm. of dry tissue and only trace quantities of potassium. The calcium content averaged 250 mg. per gm. of dry bone. Expressed in terms of calcium content, the amount of sodium in bone cortex averaged 1.15 mEq. per gm. of calcium. The sodium and chloride content of tendon were almost equivalent, averaging 1.91 and 1.99 mEq. per gm. of dry tendon, respectively. As in bone, the potassium content of tendon was extremely low. Muscle contained much larger quantities of potassium, averaging 0.411 mEq. of potassium per gm. of dry muscle, whereas the sodium and chloride content averaged 0.077 and 0.052 mEq. per gm., respectively. The mean water content of whole bone cortex, tendon and muscle equalled 18.1, 56.0 and 75.6 per cent, respectively. Plasma and red cell water electrolyte concentrations in the normal rat are included in Table I.

During the four-hour period of equilibration subsequent to the administration of the hypotonic ammonium chloride load in group I, approximately 2/3rds of the administered chloride was absorbed, the remaining peritoneal fluid becoming isosmotic with plasma. An average of 2.01 mEq. of chlo-

[3] The absence of any detectable calcium effect on the sodium readings probably relates to the fact that a city ordinance forbids the presence of propane tanks in hospitals. The considerably lower temperatures obtained in our photometer with city gas reduces the volatilization of the calcium and thereby eliminates its interfering effect.

TABLE I *

Normal rats (20)

	Bone cortex	Tendon	Muscle		Plasma	Red blood cells
Sodium (mEq./gm. of dry tissue)	*.288*	*.191*	*.077*	*Sodium* (mEq./L.)	*142*	*15.3*
Std. Deviation	.013	.008	.007	Std. Deviation	4	3.0
Std. Error †	.003	.002	.002	Std. Error	1	0.8
Chloride (mEq./gm. of dry tissue)	*.033*	*.199*	*.052*	*Chloride* (mEq./L.)	*106*	*55.3*
Std. Deviation	.006	.008	.004	Std. Deviation	4	5.5
Std. Error	.002	.002	.001	Std. Error	0.8	1.8
Potassium (mEq./gm. of dry tissue)	*.02*	*.03*	*.411*	*Potassium* (mEq./L.)	*4.02*	*87.8*
Std. Deviation			.020	Std. Deviation	0.4	7.8
Std. Error			.005	Std. Error	0.1	2.6
Water (% of wet weight)	*18.1*	*56.0*	*75.6*	*Bicarbonate* (mEq./L.)	*28.0*	
Std. Deviation	1.5	1.7	0.4	Std. Deviation	3.5	
Std. Error	0.4	0.4	0.1	Std. Error	1.0	
Calcium (mg./gm. of dry bone)	*250.3*			*pH*	*7.38*	*Hematocrit 48%*
Std. Deviation	21.0			Std. Deviation	0.07	
Std. Error	5.0			Std. Error	0.2	

* The italicized data represent the mean figure for the entire group of rats.
† Standard error denotes standard error of the mean. Any two means which differ by more than two times the standard error are significantly different (p value less than .05).

ride was therefore transferred into the rat of which 0.61 mEq. was excreted in the urine (Table II). The net chloride gain averaged 1.39 mEq. in this group (Table II). Throughout the same period, an average of 1.87 mEq. of sodium was transferred into the peritoneal fluid and approximately 0.11 mEq. was lost in the urine. The net sodium loss averaged 1.97 mEq. (Table II). In this group, therefore, the acidotic stimulus included a net gain of about 1.39 mEq. of chloride and a net loss of 1.97 mEq. of sodium. In group II, subjected to the hypertonic acidotic stimulus, the absorption of the administered chloride was virtually complete with no fluid remaining in the peritoneum after four hours. Accordingly, an average of 3.7 mEq. of chloride was transferred into the rat. The urinary chloride loss equalled 0.3 mEq. in this group, the net gain of chloride therefore averaging 3.4 mEq. (Table II). Virtually no sodium moved into the peritoneum in this group and the urinary sodium loss was barely detectable. The acidotic stimulus in group II therefore comprised almost entirely a net chloride gain of approximately 3.4 mEq. This amount equalled the combined chloride gain and sodium loss in group I.

TABLE II *

Acidotic stimuli

	Sodium lost (*mEq.*)			Chloride retained (*mEq.*)		
Group No.	Into peritoneum	Urinary	Total	From peritoneum	Urinary loss	Net gain
I	*1.87*	*.11*	*1.97*	*2.01*	*.61*	*1.39*
II	*0*	*.03*	*.03*	*3.70*	*.30*	*3.40*

* The italicized data represent the mean figure for the entire group of rats.

In group I the net water gain averaged 5 cc., whereas, in group II, urine output was so low that the net water gain averaged 12 cc.

Both groups of acidotic rats revealed a comparable degree of acidosis as reflected in the fall of plasma pH to 7.17 and 7.19, respectively (Tables III and IV). In group I the plasma electrolyte pattern was characterized by a 13 mEq. per L. or 9 per cent fall in the plasma sodium con-

TABLE III *

Group I—Acidotic rats (18)—Hypotonic stimulus

	Bone cortex		Tendon		Muscle			Plasma		Red blood cells	
Sodium (mEq./gm. of dry tissue)	*.260*	(.288)	*.159*	(.191)	*.051*	(.077)	*Sodium* (mEq./L.)	*129.0*	(142)	*12.8*	(15.3)
Std. Deviation	.013		.012		.005		Std. Deviation	6.0		3.5	
Std. Error †	.003		.003		.001		Std. Error	1.2		0.9	
Chloride (mEq./gm. of dry tissue)	*.037*	(.033)	*.201*	(.199)	*.045*	(.052)	*Chloride* (mEq./L.)	*107.0*	(106)	*64.3*	(55.3)
Std. Deviation	.007		.013		.004		Std. Deviation	7.0		7.5	
Std. Error	.002		.003		.001		Std. Error	1.5		2.0	
Potassium (mEq./gm. of dry tissue)	*.03*	(.02)	*.03*	(.03)	*.415*	(.411)	*Potassium* (mEq./L.)	*4.9*	(4.02)	*77.2*	(87.8)
Std. Deviation					.02		Std. Deviation	0.7		5.5	
Std. Error					.005		Std. Error	0.2		2.0	
Water (% of wet weight)	*17.9*	(18.1)	*56.0*	(56.0)	*76.3*	(75.6)	*Bicarbonate* (mEq./L.)	*12.2*	(28.0)		
Std. Deviation	1.8		1.5		0.7		Std. Deviation	1.3			
Std. Error	0.4		0.5		0.15		Std. Error	0.4			
Calcium (mg./gm. of dry bone)	*249.5*	(250.3)					*pH*	*7.17*	(7.38)	*Hematocrit* *51%*	(48%)
Std. Deviation	21.0						Std. Deviation	0.09			
Std. Error	5.0						Std. Error	0.03			

* The data in italics represent the mean figure for the entire group of rats; the data in parentheses represent the mean data from the normal rats.
† Standard error denotes standard error of the mean. Any two means which differ by more than two times the standard error are significantly different (p value less than .05).

centration, a fall of 16 mEq. per L. in the bicarbonate concentration, but no change in the chloride concentration (Table III). In group II, the acidosis was characterized by an increase in the plasma chloride concentration of 9 mEq. per L. or 9 per cent, a very slight fall in plasma sodium concentration, and a fall of about 8.5 mEq. per L. in the bicarbonate level (Table IV).

Despite the difference in the plasma electrolyte pattern between each acidotic group, namely a sodium bicarbonate deficiency in group I as contrasted to a relative chloride excess in group II, the changes in the electrolyte content of bone and tendon in both these groups were very similar (Tables III and IV). The bone sodium content fell 10 and 9 per cent or 0.028 and 0.025 mEq., re-

TABLE IV *

Group II—Acidotic rats (12)—Hypertonic stimulus

	Bone cortex		Tendon		Muscle			Plasma		Red blood cells	
Sodium (mEq./gm. of dry tissue)	*.263*	(.288)	*.162*	(.191)	*.058*	(.077)	*Sodium* (mEq./L.)	*139*	(142)	*18.2*	(15.3)
Std. Deviation	.015		.005		.004		Std. Deviation	9.0		4.6	
Std. Error †	.005		.002		.001		Std. Error	3.0		1.5	
Chloride (mEq./gm. of dry tissue)	*.038*	(.033)	*.196*	(.199)	*.051*	(.052)	*Chloride* (mEq./L.)	*115*	(106)	*63.2*	(55.3)
Std. Deviation	.006		.011		.002		Std. Deviation	7.0		6.0	
Std. Error	.002		.003		.0006		Std. Error	2.0		2.0	
Potassium (mEq./gm. of dry tissue)	*.02*	(.02)	*.03*	(.03)	*.411*	(.411)	*Potassium* (mEq./L.)			*81.1*	(87.8)
Std. Deviation					.025		Std. Deviation			6.6	
Std. Error					.008		Std. Error			2.2	
Water (% of wet weight)	*16.8*	(18.1)	*55.7*	(56.0)	*77.0*	(75.6)	*Bicarbonate* (mEq./L.)	*19.5*	(28.0)		
Std. Deviation	1.8		0.8		0.4		Std. Deviation				
Std. Error	0.6		0.3		0.2		Std. Error				
							pH	*7.19*	(7.38)	*Hematocrit*	
							Std. Deviation	0.07		46%	(48%)
							Std. Error	0.03			

* The data in italics represent the mean figure for the entire group of rats; the data in parentheses represent the mean data from the normal rats.
† Standard error denotes standard error of the mean. Any two means which differ by more than two times the standard error are significantly different (p value less than .05).

spectively, in groups I and II with little change in chloride potassium or calcium content. Tendon sodium content fell 17 per cent and 15 per cent or 0.032 and 0.029 mEq. in groups I and II, respectively, without any apparent change in chloride or potassium content. The water content of these tissues did not change in the acidotic rats.

The changes in electrolyte content of muscle in groups I and II were not identical (Tables III and IV). Muscle sodium content fell 34 per cent or 0.026 mEq. in group I and 25 per cent or 0.019 mEq. in group II. The chloride content fell 15 per cent or 0.007 mEq. in group I but did not change in group II. No detectable changes from the normal were apparent in the potassium content of groups I and II. The water content, however, increased slightly in both acidotic groups. The changes in red cell water electrolyte composition are presented in Tables III and IV.

DISCUSSION

The ratio of 9/1 for the sodium and chloride content of normal bone cortex when compared to the ratio of these ions in an ultrafiltrate of serum (1:2/1), indicates that at least 87 per cent of the bone sodium cannot be attributed to extracellular fluid and is probably confined by the bone cortex. Whether this considerable fraction of bone sodium is confined by the calcium (apatite) crystal or by the underlying protein matrix is not proved. It has been suggested that sodium is present as a double carbonate salt within the apatite crystal (16, 17). The very low potassium content of bone cortex and its well known sparsity of cells renders the possibility of cellular disposition for this excess sodium extremely unlikely. Compared to bone, dense connective tissue, as represented by tendon, contains a considerable quantity of chloride as well as sodium. Previous workers have concluded that a considerable fraction of tendon chloride cannot be attributed to its extracellular fluid phase (7, 8). In these data the ratio of chloride/sodium in tendon exceeds that of a plasma ultrafiltrate (1.04/.83) by approximately 25 per cent suggesting that at least 25 per cent of the total tendon chloride is confined by the underlying tissue. It is conceivable, however, that a fraction of the tendon sodium is also confined by the tissue matrix. Under this circumstance, the excess chloride of tendon would exceed 25 per cent of the total chloride content. That such is the case is suggested by dividing the sodium and chloride content of tendon by its water content. (Since water comprises 56 per cent of whole tendon (Table I), approximately 1.27 cc. of water is present per gm. of dry tendon.) This calculation yields a theoretical concentration of 150 mEq. per L. of tendon water for sodium and 156 mEq. per L. for chloride. In so far as these concentrations exceed those of interstitial fluid by approximately 5 and 30 per cent, at least these fractions of the tendon sodium and chloride, respectively, cannot be attributed to interstitial fluid. If a fraction of tendon water is contained within cells or bound in some way and therefore removed from extracellular fluid, the percentage of sodium and chloride confined by this tissue would exceed these last mentioned figures. The trace quantities of potassium and the sparsity of cells in tendon suggest that these excess quantities of chloride and sodium are not confined within the few tendon cells but by the underlying protein matrix of the dense connective tissue.

The fall in bone sodium in acidotic groups I and II, 0.028 and 0.025 mEq. per gm. of dry bone, respectively (Tables III and IV), far exceeds that fall which may have been anticipated from the change in extracellular fluid composition. In group I, the 9 per cent fall in plasma sodium concentration without any reduction in the chloride space of bone should have produced a maximal decrease in bone sodium content of about 0.003 mEq. per gm. In group II, no distinct change in plasma sodium concentration or in chloride space of bone was evident. Accordingly, it may be concluded that the acidotic stimulus in each group effected a transfer of 0.025 mEq. of sodium per gm. of dry bone into the extracellular fluid (Figure 1). This transfer occurred without any detectable change in bone calcium or water content in group I and with only a very slight, probably insignificant fall, in bone water in group II (Tables III and IV).

Assuming that all of the tendon sodium is attributable to the extracellular fluid of tendon, a fall of 9 per cent in plasma sodium concentration should have produced in the group I rats a proportionate fall in tendon sodium content, or a fall of 0.017 mEq. per gm. The reduction observed, however, averaged 0.032 mEq. almost twice the expected fall (Table III). In group II, the 8

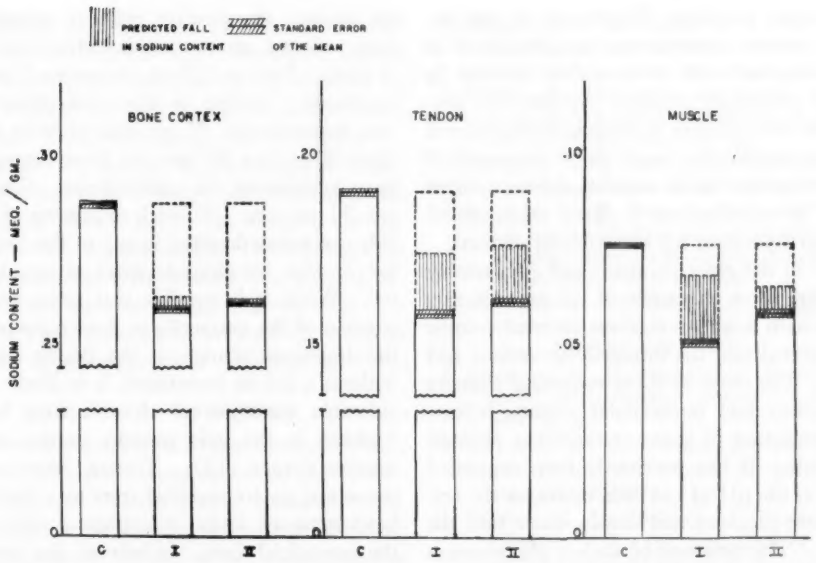

FIG. 1. A GRAPHIC REPRESENTATION OF THE FALL IN TISSUE SODIUM AFTER ACUTE ACIDOSIS

The shaded area represents the expected fall in tissue sodium. The clear area denotes that quantity of sodium per gm. of dry tissue transferred into the fluid phase of the extracellular volume.

per cent increase in plasma chloride concentration without any change in tendon chloride content presumably reflects a proportionate decrease in extracellular phase of tendon. Based upon the above mentioned assumption, such a fall in extracellular fluid volume should have produced a comparable reduction in tendon sodium content, of 0.015 mEq. per gm. As in group I, however, the observed decline, 0.029 mEq. was twice that anticipated on this premise. These excessive falls in tendon sodium are not compatible with the assumption that the sodium content of tendon is entirely attributable to its content of extracellular fluid. Instead these data in the acidotic rats suggest, as do the comparisons of the sodium and water content of normal rat tendon, that at least 7 to 8 per cent of tendon sodium, about 0.015 mEq. per gm. of dry tendon is confined in the tissue matrix of tendon and may be released to the extracellular fluid after an appropriate stimulus (Figure 1). The sole alternate explanation for this excessive drop in tendon sodium content would demand a considerable undemonstrated reduction in extracellular fluid volume of dense connective tissue. The absence of a coincidental and consistent fall in tendon water or chloride content renders this view unlikely.

The falls in muscle sodium content, namely 34 and 25 per cent, or 0.026 and 0.019 mEq. per gm. in acidotic groups I and II, respectively (Tables III and IV), are best explained by a combination of changes. In both groups, a distinct fall in extracellular volume of muscle, as measured by the chloride space, was detected. In group I this decrease averaged 13 per cent (a 13 per cent fall in muscle chloride content without change in plasma chloride concentration); in group II, it averaged 8 per cent (an increase in plasma chloride concentration of 8 per cent without change in muscle chloride content). In group I an additional 9 per cent fall in muscle sodium content may be attributed to the reduced plasma sodium concentration. The falls in muscle sodium content, however, exceeded that attributable to the changes in the extracellular fluid of muscle by about 12 per cent or 0.009 mEq. per gm. in group I and 17 per cent or 0.013 mEq. per gm. in group II (Figure 1). It may be concluded that approximately 0.011 mEq. of sodium per gm. of dry muscle was transferred out of cells by these acidotic stimuli.

Such a transfer involving 75 per cent of total intracellular sodium content was not associated in these experiments with a detectable increase in the muscle potassium content. While it is conceivable that the quantity of potassium transferred was proportionately too small to be measured, it is possible that the fall in cellular sodium content may best be explained as a direct exchange of extracellular hydrogen for intracellular sodium.

The fall in the chloride space and presumably extracellular phase of muscle of the acidotic rats coexistent with a slight increase in total muscle water suggested that the intracellular volume had expanded. The cause of these reciprocal changes in extracellular and intracellular volume following the production of acute extracellular acidosis is not certain. It has previously been suggested that a fall in the pH of red cells increases the cellular osmotic pressure and draws water into the cells (18). The presence of such a phenomenon in muscle cells would best explain the simultaneous reduction in extracellular volume and expansion of intracellular volume.

The stimulus responsible for the transfer of sodium out of bone cortex, dense connective tissue and muscle appeared to be the consistent fall in plasma pH, reflecting a state of severe decompensated acidosis. The alternate stimuli including a negative sodium balance, reduced plasma sodium concentration, increased plasma chloride concentration, or a marked fall in bicarbonate concentration were not consistently present in both acidotic groups. For reasons mentioned above it is unlikely that the sodium derived from bone and tendon came from cellular sources. It is probable that a fall in extracellular pH so alters the underlying protein matrix or the apatite crystal of bone as to effect a release of the confined sodium. That such a change in pH might similarly affect the protein molecules in muscle cells also seems feasible.

The relative amounts of sodium released 0.025 mEq., 0.015 mEq., and 0.010 mEq. per gram of bone, tendon and muscle, respectively, also appear relevant (Figure 1). If it be assumed that comparable changes occur in man after a comparable stimulus bone, connective tissue, and muscle cells would release 175, 75 and 70 mEq., respectively, to the fluid phase of the ECV.

Presuming that changes in the over-all chloride space serve as an index of changes in extracellular volume, the positive chloride balance should have reflected an increase in extracellular volume of about 15 cc. and 30 cc. in groups I and II, respectively. Insofar as the extracellular fluid of rats approximates 20 per cent of body weight or about 60 cc. in a 300 gm. rat, these changes should have augmented the extracellular volume by 25 and 50 per cent. No such expansion of the chloride space was detected in any of the tissues studied: in fact, the chloride space of muscle actually fell. While it is possible that some over-all expansion of the extracellular fluid occurred despite the discrepant changes in the tissues studied and without a fall in hematocrit, it is likely that considerable quantities of chloride may have been confined in the solid protein matrix or cells of specific tissues (19). Viscera, loose connective tissue and gastrointestinal tract may, for example, have removed large quantities of chloride from the interstitial fluid. Whatever the precise disposition of this "lost chloride," it may be concluded that an over-all expansion of the chloride space may not necessarily indicate a comparable expansion of extracellular volume and need not be reflected in any individual tissue—even one present in such large mass as muscle. Conversely, conclusions drawn from changes in the chloride space of muscle may not be applied to the organism as a whole.

The quantities of sodium confined in bone and tendon with relatively little water and the release of considerable fractions of this base without any detectable changes in water content suggest that the osmotic activity of the confined sodium is considerably reduced. Upon transfer to the free extracellular fluid the osmotic work produced by these ions may be considerably enhanced.

These data further support the contention that the extracellular compartment comprises several heterogeneous phases rather than one homogeneous solution. In addition to the fluid phase or interstitial fluid with uniform electrolyte composition, there are collections of trans-cellular fluids (20) with varying electrolyte compositions: *i.e.,* cerebrospinal fluid, ocular fluid, gastrointestinal juices. Furthermore there exist relatively solid phases composed primarily of protein matrix which are interspersed throughout the interstitial fluid and are concentrated in characteristic sites such as bone and connective tissue. These solid phase tissues appear capable of con-

fining variable quantities of sodium and chloride (21). Such electrolytes, as in the case of sodium in these experiments, may be rapidly released by extreme changes in the composition of the interstitial fluid. The circumstance that the various extracellular phases respond in a heterogeneous and discrepant fashion to a single stimulus precludes the possibility that a single ion or molecule can distribute itself homogeneously throughout the entire extracellular compartment and thereby afford a precise measure of its total volume. At best, such tracer substances provide an index of the volume of the fluid phase or interstitial fluid.

SUMMARY AND CONCLUSIONS

1. Electrolyte analyses of bone cortex, tendon, and muscle from the normal rat reveal the following composition. Bone cortex contains 0.288 mEq. of sodium, 0.033 mEq. of chloride and 0.02 mEq. of potassium per gm. of dry tissue; tendon 0.191 mEq. of sodium, 0.199 mEq. of chloride and 0.03 mEq. of potassium per gm. of dry tissue; and muscle 0.077 mEq. of sodium, 0.052 mEq. of chloride and 0.411 mEq. of potassium per gm. of dry tissue. The water content of whole bone cortex, tendon, and muscle averages 18.1 per cent, 56 per cent and 75.6 per cent, respectively.

2. These data indicate that approximately 87 per cent of bone sodium, at least 7 per cent of tendon sodium and 33 per cent of tendon chloride, and approximately 20 per cent of muscle sodium cannot be attributed to the extracellular fluid content of these tissues.

3. Severe acute extracellular acidosis mobilizes 0.025 mEq., 0.015 mEq. and 0.010 mEq. of sodium per gm. of tissue from the non-extracellular fluid stores of bone, tendon, and muscle, respectively.

4. Acute acidosis evokes a reduction in extracellular volume and a coexistent expansion of intracellular volume in muscle.

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CHROMATOGRAPHIC PURIFICATION OF HUMAN SERUM ACCELERATOR GLOBULIN [1]

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There appears to be general agreement among investigators of blood clotting regarding the existence in plasma of a heat-labile factor which potentiates the action of thromboplastin as a prothrombin converting agent (1). This clotting agent appears to be a protein which is not adsorbed on barium sulfate and which will correct the prolonged prothrombin time of aged, oxalated, human plasma. There is a lack of agreement about its nomenclature; the terms accelerator globulin (2), proaccelerin (3), Factor V (4), and labile factor (5) being used predominately.

Considerable confusion exists about the presence and characteristics of accelerator globulin in serum. This is partly due to the fact that another accelerator has been identified in serum which can be readily removed by barium sulfate adsorption. This factor is most frequently referred to as SPCA (6), proconvertin (7), stable factor (8) or co-thromboplastin (9).[2] Barium sulfate treatment of bovine serum does not remove the accelerator globulin (AcG) activity. In fact, after barium sulfate adsorption, considerably more AcG activity is demonstrable in bovine serum than in bovine plasma. Similarly, it is possible to produce a significant increase in plasma AcG activity by treatment of bovine plasma with purified thrombin. To explain these phenomena, it was proposed by Ware, Murphy, and Seegers (11) that thrombin causes activation of AcG. Two forms of AcG were suggested to exist, plasma AcG and serum AcG. It seems probable that the AcG in plasma exists in a relatively inert form and that serum AcG results from the action of thrombin on the plasma precursor (11).

Proposal of the existence of two forms of AcG with thrombin as mediator has been accepted by some investigators. Owren's proaccelerin is synonymous with plasma AcG; accelerin, with serum AcG (12). Others have not accepted this hypothesis on the basis that the accelerator activity produced with thrombin is probably due to factors other than AcG (13, 14). This is not surprising because of the complex reactions involved and because a different situation exists in human serum. There appears to be minimal AcG activity in human serum, all of which is removed with barium sulfate.

The AcG activity in human plasma and serum was investigated recently by Lewis and Ware (15). They found that thrombin activation of human plasma AcG proceeded in much the same manner as bovine. However, shortly after addition of the thrombin, the serum AcG activity diminished to a level which was less than that of the original plasma. This was apparently the result of an inhibitor which was isolated in crude form. Furthermore, they demonstrated that purified plasma AcG, of human origin, which had been adsorbed by barium sulfate, developed a 10 to 30-fold increase in activity on incubation with small amounts of purified thrombin. Since the thrombin had also been subjected to barium sulfate adsorption this experiment removes any reasonable doubt that the activation of plasma AcG by thrombin is due to the evolution of SPCA or convertin.

Thus, it appears that thrombin activates AcG of both bovine and human origins. Plasma precursors from both species as well as the activated bovine serum AcG are not adsorbed appreciably on barium sulfate. However, serum AcG of hu-

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[2] These and other synonyms have been listed by the Subcommittee on Nomenclature of Nonprothrombin Plasmin Factors of the Josiah Macy, Jr. Foundation (10).

man origin is readily adsorbed. Therefore, it seems that in the human species, plasma AcG and serum AcG parallel the adsorption characteristics of thrombin and prothrombin, respectively.

Some success has been achieved in this laboratory in the isolation of human plasma AcG (16) but the products obtained were of extreme lability, low specific activity and did not lend themselves to adequate study of further purification procedures. In this communication, a method is described for the isolation of serum AcG of high specific activity by chromatography on an ion exchange resin and additional evidence is provided for the activation of AcG by thrombin.

METHODS AND MATERIALS

Assay methods

Accelerator globulin. AcG activity was measured by the one-stage method of Lewis and Ware (17). It is defined as the heat-labile property of plasma which accelerates the clotting of aged, oxalated human plasma in the presence of tissue thromboplastin and calcium ions. An arbitrary unit of AcG activity was selected which is equivalent to that displayed by 0.01 ml. of freshly-collected, citrated human plasma. Bovine plasma has been found to exhibit eight to nine times this amount of activity.

Thrombin. Thrombin was assayed by measuring the clotting time of fibrinogen standardized according to that used in the two-stage analysis of prothrombin (18).

Protein. Protein was measured by the modified Folin-Ciocalteu method of Daughaday, Lowry, Rosebrough, and Fields (19).

Materials

Thrombin preparations. Parke, Davis & Company, Thrombin Topical, was reconstituted and rendered free of prothrombin by adsorption with barium sulfate (15). Following this, the solutions were made up to 250 units per ml. in 50 per cent glycerol and stored at 0° C. Prior to use, the thrombin was diluted with 0.9 per cent sodium chloride to give the desired activity.[3] These crude thrombin products were employed in the majority of our preparative procedures for the activation of AcG.

Purified thrombin of human and bovine origin was employed in some preparations. Human thrombin was obtained by activation of purified human prothrombin products (16) with 25 per cent sodium citrate (20). Bovine thrombin was derived by similar methods from a purified prothrombin preparation (No. 530818 having approximately 2,530 2-stage units per mg. tyrosine) kindly supplied by Dr. Walter H. Seegers of Wayne University Medical School, Detroit. The reaction products obtained by activation of prothrombin with sodium citrate were further purified by zone electrophoresis on a starch block (21) from which a single electrophoretic component having thrombin activity was isolated.

Preparation of resin and column. Rohm and Haas Amberlite IR-400, a strongly basic polystyrene quaternary amine type resin having a mesh size of 16–30 and charged in the chloride cycle, was employed for the chromatographic separation and purification of serum AcG. One hundred grams of anion resin, without prior purification, were triturated with approximately 1 liter of an aqueous 10 per cent solution of sodium chloride for 1 hour. After decantation of the salt solution, the resin was washed with several liters of distilled water until the washings were free of chloride ion. This process was repeated and the charged resin stored in the moist state until ready for use. Amberlite IR-400 anion resins with three different lot numbers were employed in the experiments to be described and all proved satisfactory.

The chromatographic column was prepared by pouring an aqueous suspension of resin into a 1.2×36 cm. glass column fitted with a small quantity of glass wool at the tapered end to prevent leakage of the resin. The glass column was filled with resin to a height of 24 cm. and was then washed with 10 ml. portions of water to insure uniform packing. This procedure permitted a relatively constant flow time through the resin and no detectable bubble formation. It was found desirable to pack the top of the column with approximately 2 mm. of finely meshed glass beads to prevent agitation of the uppermost resin particles by the influent. The hold-up volume of the resin was approximately 7 to 8 ml.

Regeneration of the resin was accomplished by triturating 100 gm. of IR-400 with 1 L. of 6 N sodium hydroxide, washing copiously with water until the wash solution approximated a neutral pH, and recharging with 10 per cent sodium chloride in the manner described above.

Preparation of crude plasma AcG. Crude plasma AcG was prepared by a modification of the method of Lewis and Ware (16) with the omission of the salt fractionation procedures. Blood was taken from putatively normal donors by venipuncture and mixed immediately in a proportion of 9 to 1 with 0.1 M sodium citrate. After separation of the cells by centrifugation at 2,500 G. for 10 minutes, the supernatant plasma was rendered free of prothrombin by successive adsorptions on barium citrate and barium sulfate precipitates. In this technic, 2 ml. of 1.0 M barium chloride were added slowly to 20 ml. of plasma with constant agitation at 0° C. After standing in the cold for 10 minutes, the barium citrate precipitate was removed by centrifugation at 0° C. for 15 minutes at 33,000 G. to the supernatant fluid thus obtained, 2 ml. of a 1.0 M solution of sodium sulfate

[3] Ampoules of Parke-Davis Topical Thrombin are guaranteed to contain *at least* a total of 5,000 Iowa units. In most instances they contain appreciably more as well as large amounts of unconverted prothrombin, thromboplastin, calcium and extraneous protein. However, it was found to be of sufficient quality for use in the procedure to be described.

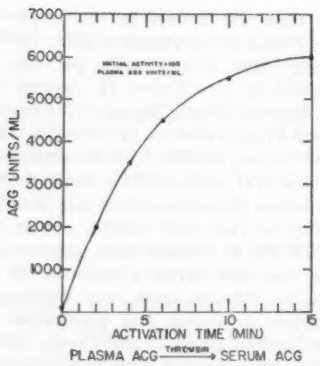

FIG. 1. THE ACTIVATION OF PLASMA AcG TO SERUM AcG IN THE ACID PRECIPITATE BY ADDITION OF THROMBIN

To 10 ml. of acid precipitate at 37° C., 1 ml. of thrombin solution containing 50 units per ml. was added. At intervals, 0.1-ml. aliquots of the incubation mixture were taken and tested for AcG activity. (The points on the above curve represent the average of twelve experiments.)

were added slowly in the cold and the barium sulfate subsequently separated by high speed centrifugation.

After this treatment with barium citrate and barium sulfate, the plasma obtained was devoid of detectable amounts of prothrombin but contained most of its plasma AcG and fibrinogen, as well as antihemophilic globulin and plasminogen. It was then diluted 20 times with distilled water at room temperature and adjusted to pH 5.5 with the slow addition of 1 per cent acetic acid and constant stirring. The precipitate formed was harvested by centrifugation in the cold for 10 minutes at high speed and was subsequently dissolved in one-half of its original plasma volume with 0.9 per cent sodium chloride, pH 7.0 to 7.2. This material will be identified henceforth in this paper as the acid precipitate.

The acid precipitate obtained in the above manner was usually found to contain approximately 50 per cent of the original AcG activity of the starting plasma (*i.e.*, 100 units per ml.) and had a protein concentration of 6 to 8 mg. per ml.

RESULTS

Activation of plasma AcG in the acid precipitate

The activation of plasma AcG to serum AcG by incubation of the acid precipitate with thrombin is diagrammed in Figure 1. Upon the introduction of thrombin to the acid precipitate, fibrinogen clotted out. The fibrin thus formed was removed mechanically. With most preparations, the AcG activity was increased 50 to 60 times by the addition of thrombin at a concentration of 5 units per ml.

After 15 to 20 minutes of activation, the AcG activity reached a maximum and thereafter decreased appreciably. The drop in activity is probably due to the fact that thrombin not only elaborates a more active accelerator but also destroys it (22). This was found to be particularly true when concentrations of thrombin greater than 5 units per ml. of acid precipitate were used or when highly active electrophoretically purified thrombin preparations were employed (Figure 2). Fortunately for our purposes, the thrombin destruction of AcG was found to be minimized when the incubation mixture was subjected to chromatographic separation of serum AcG.

Chromatographic separation and purification of serum AcG

After the AcG activity of the incubation mixture reached a maximum, the crude serum AcG product thus obtained had an activity of 5 to 6,000 units per ml. and a protein concentration of approximately 5 to 6 mg. per ml. It was immediately placed on the Amberlite IR-400 column and allowed to percolate through the resin at a flow rate of approximately 1.5 ml. per min. The column was then washed with distilled water or with 0.9 per cent sodium chloride, pH 6.0, until the eluates gave negative tests for protein and AcG activity. A volume of wash solution equivalent to the initial volume placed on the column was found to be sufficient for this purpose. Resolution of the AcG activity was achieved by the addition of 3.0

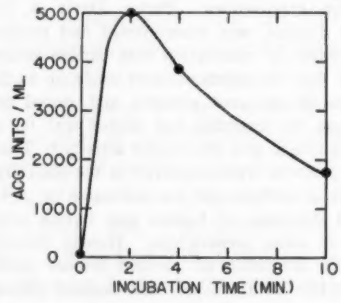

FIG. 2. ACTIVATION OF ACID PRECIPITATE WITH HUMAN THROMBIN

Conditions of the experiment similar to Figure 1. Two hundred units of human thrombin, derived by citrate activation of purified human prothrombin, were added to 10 ml. of the incubation mixture.

The initial AcG activity was 100 units per ml.

per cent sodium chloride to the resin column. All effluents were collected as 1-ml. samples and tested for AcG activity as well as protein. Two typical experiments are presented in Figures 3 and 4.

It is obvious from Figure 3 that a large portion of the protein and some AcG activity pass through the column and are washed out with water. Upon introduction of 3 per cent sodium chloride to the influent, a front of AcG activity with only minimal amounts of protein becomes apparent. In this experiment there are two protein components associated with the second peak while the AcG activity appears to reside somewhere between them. When the column is first washed with 0.9 per cent sodium chloride, one of these peaks of protein is eliminated and further purification of AcG results (Figure 4). However, the AcG obtained in this fashion is still contaminated with traces of extraneous protein since chromatography of the unactivated acid precipitate itself results in a small amount of protein in the second peak (Figure 5). Attempts to gain further removal of this contaminant by chromatography of the acid precipitate

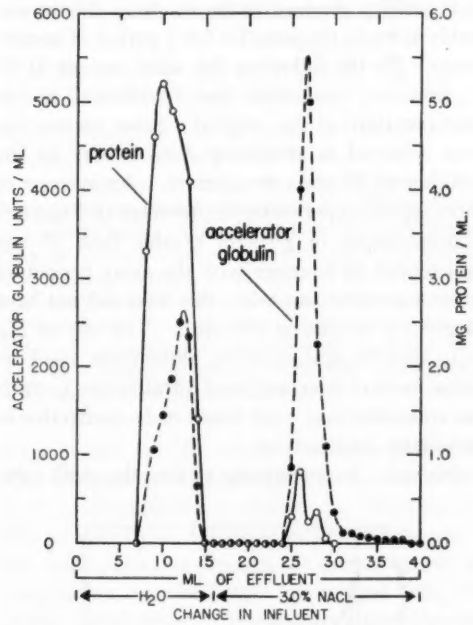

FIG. 3. CHROMATOGRAPHIC SEPARATION OF SERUM ACG FROM THE ACID PRECIPITATE EMPLOYING WATER IN THE INITIAL WASH SOLUTION

Solution before chromatography

Volume	7.0 ml.	Protein/ml.	4.94
AcG units/ml.	5,750	Specific activity	1.160

Effluent volumes

	First peak	*Second peak*
Volume	1 ml. (No. 12)	1 ml. (No. 27)
AcG units/ml.	2,500	5,000
Protein/ml.	4.7	0.28
Specific activity	532 units/mg. protein	17,850

Total recovery

Protein	88%
Activity	68%

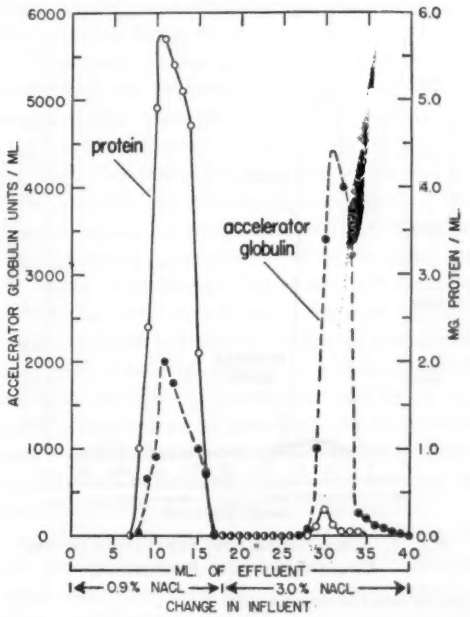

FIG. 4. CHROMATOGRAPHIC SEPARATION OF SERUM ACG FROM THE ACID PRECIPITATE EMPLOYING 0.9 PER CENT SODIUM CHLORIDE IN THE INITIAL WASH SOLUTION

Solution before chromatography

Volume	8 ml.	Protein/ml.	5.5
AcG units/ml.	6,150	Specific activity	1.118

Effluent volumes

	First peak	*Second peak*
Volume	1 ml. (No. 11)	1 ml. (No. 32)
AcG units/ml.	2,000	4,000
Protein/ml.	5.7	0.05
Specific activity	351 units/mg. protein	80,000 units/mg. protein

Protein	75%
Activity	50%

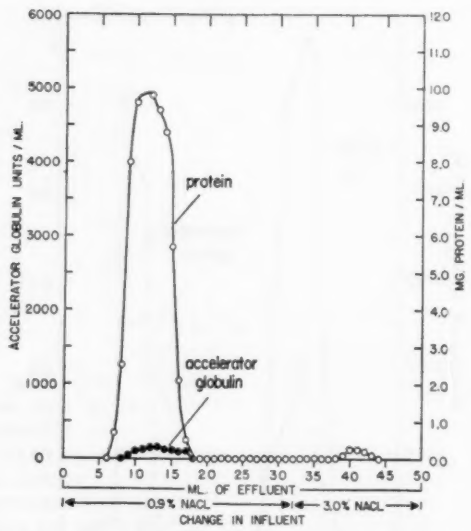

FIG. 5. CHROMATOGRAPHIC SEPARATION OF THE UNACTIVATED ACID PRECIPITATE

Solution before chromatography

Volume	8 ml.	Protein/ml.	13.0 mg.
AcG units/ml.	125	Specific activity	10

Effluent volumes

	First peak	*Second peak*
Volume	1 ml. (No. 12)	1 ml. (No. 41)
AcG units/ml.	145	0
Protein/ml.	9.8	0.22
Specific activity	15 units/ml.	—

prior to thrombin activation and rechromatography have not been successful.

Some chemical and physical properties of human serum AcG

Ultra-violet absorption. Products obtained by the above chromatographic procedure were examined for ultra-violet absorption by employing a Beckman Model DU Quartz Spectrophotometer. Solutions of serum AcG in 0.1 N NaOH and in 0.1 N HCl gave an ultraviolet absorption curve between 320 and 220 mu characteristic of a protein or polypeptide having a tyrosine peak between 275 to 280 mu.

Electrophoresis. Samples of human serum AcG having high specific activity were subjected to zone electrophoresis on filter paper using the method of Kunkel and Tiselius (23). Electrophoresis was carried out in 0.05 M veronal buffer, pH 8.6, $\mu = 0.05$ for 20 hours at 0° C. under a gradient of 8 volts per cm. of paper. After drying, the strips were stained with 1 per cent bromphenol blue in ethyl alcohol saturated with mercuric chloride and then washed with 5 per cent acetic acid. Under these conditions, the majority of the material in the AcG sample migrated as one component with a mobility between that of beta and gamma globulin. Attempts to elute the AcG activity after sectioning the filter paper strip into 1 cm. sections were unsuccessful.

Stability. After column chromatography, the AcG activity obtained in the resulting eluates was stable at room temperature for a period of several hours. By the following day after storage at 0° C., however, the activity had deteriorated to less than one-third of the original. Some success has been achieved in stabilizing AcG activity by the addition of 50 per cent glycerol. An average of three typical experiments is presented in Figure 6. Concentrations of glycerol greater than 50 per cent tended to interfere with the assay procedure while concentrations below this level did not have as great a stabilizing influence. The use of N_2, CO_2, ascorbic acid, cysteine, glutathione, tosyl arginine methyl ester and beef ultrafiltrates in various concentrations were found to be ineffective in stabilizing AcG activity.

Dialysis. In attempting to free the AcG solu-

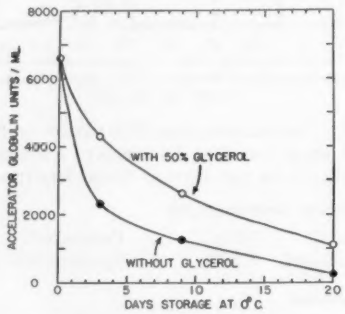

FIG. 6. THE STABILITY TO STORAGE AT 0° C. OF HUMAN SERUM AcG IN THE PRESENCE AND ABSENCE OF GLYCEROL

Effluent samples of high specific activity were obtained from three different chromatographic preparations. These were divided into controls and samples containing 50 per cent glycerol (v/v) and stored in a refrigerator at 0° C. Aliquots were taken and assayed for AcG activity at the time indicated.

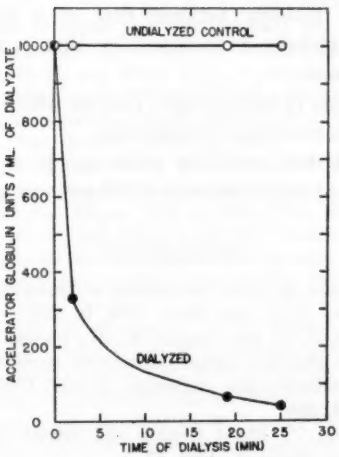

FIG. 7. THE EFFECT OF DIALYSIS ON HUMAN SERUM ACG ACTIVITY

Three samples of chromatographically isolated AcG were pooled and diluted with 0.9 per cent sodium chloride to a volume having an activity of 1,000 AcG units per ml. Five ml. of this solution were then subjected to rapid dialysis in a cellophane casing against 1 L. of 0.9 per cent sodium chloride at room temperature in a mechanical dialyzer similar in construction to one recently reported (16). At intervals, 0.2-ml. aliquots of the dialysate, as well as a control sample of undialyzed solution, were taken and assayed for AcG activity.

tions of salts by dialysis, it was observed that the activity deteriorated rapidly. A typical experiment is diagrammed in Figure 7. Whether this loss of activity is due to destruction, diffusion of the AcG molecules through the membrane, or adsorption on the membrane, has not yet been definitely determined. Preliminary investigations provide evidence that the loss of serum AcG activity upon dialysis is the result of adsorption or destruction by the dialysis membrane.

DISCUSSION

The adsorption of serum AcG of human origin to an Amberlite resin appears to be unique. There is no appreciable adsorption of the plasma precursor to the resin under any conditions that we have been able to demonstrate. This provides evidence that the precursor, plasma AcG, is inert and that the activity it exhibits is generated in the assay procedure.

It seems rather unlikely that the thrombin-generated accelerator activity can be attributed to any other clotting factor than the AcG itself. Under the conditions of these experiments, both the precursor and the thrombin have been exposed to barium sulfate adsorptions which should be adequate to remove other known accelerators. Furthermore, the thrombin continues to produce its effect on AcG even after the former has been subjected to extensive purification. Thrombin alone does not have a significant effect on the assay system in the concentration employed either before or after it has been run through the resin column. When thrombin is incubated with acid precipitated from aged human plasma with no demonstrable precursor activity, no accelerator activity is produced either before or following the resin treatment. Similar results are obtained with acid precipitates which have had their AcG activity destroyed by heating at 56° C. for 30 minutes.

Crude plasma AcG products prepared as the acid precipitate from bovine plasma and then activated with thrombin in the manner described above, demonstrate no adsorptive properties for the resin and pass through the columns. This phenomenon lends further support to the thesis that bovine and human serum AcG are different in certain of their physical and chemical properties.

The exact nature of the chromatographic process whereby serum AcG is adsorbed to the resin column and eluted with salt solution is unknown. This mechanism doubtless involves a complicated series of events. In all probability, the adsorption phenomenon results from a saturation of the column with serum AcG while plasma AcG and unadsorbed serum AcG as well as thrombin are washed from the column at low salt concentrations. The resolution of the adsorbed serum AcG activity is probably due to a gradient type of elution process in which a concentration of salt is obtained in which serum AcG is more soluble and readily favors partition into the mobile phase. Since the conditions of the experiments probably do not operate under the requirements of reversibility required of true chromatography, the process more correctly conforms to Zittle's term, adsorption filtration (24). Such a situation would hold where separation is made of components more strongly adsorbed and components not adsorbed.

The chemical and physical properties of the serum AcG products obtained by the above chro-

matographic method are indeed remarkable. They point up the fact that serum AcG is a trace substance capable of displaying a high degree of catalytic activity in amounts which almost defy chemical detection. In spite of the fact that the isolation procedure has resulted in about a thousand-fold purification of human AcG activity, it is quite likely that the products obtained are still not chemically pure. This is readily apparent in Figure 5 where it is seen that trace amounts of protein with no AcG activity are eluted from the column by 3 per cent sodium chloride. Repetitive chromatography of the final serum AcG product in attempts to achieve better purification have been unsuccessful. This is largely due to the instability of the preparation as well as the large amount of dilution necessary in the rechromatographic procedure.

It is interesting to speculate that the activated AcG of human origin may be a substance of low molecular weight, possibly approaching the dimensions of a polypeptide. This hypothetical reasoning stems from the fact that when plasma AcG is activated with thrombin, a number of fragments soluble in 5 per cent trichloracetic acid are split off the molecule concomitantly with the increase in accelerator activity (25). Also, the fact that serum AcG is adsorbed to an ion exchange resin, similar to other low molecular weight substances (24), as well as its loss of activity on dialysis, offers interesting avenues of approach in further investigations into the exact chemical nature of this substance.

SUMMARY

A method is described for the purification of human serum accelerator globulin by chromatography on Amberlite IR-400 columns. The technique involves the prior isolation of a crude plasma accelerator globulin preparation from freshly-collected, human, ACD plasma by barium citrate adsorption and acid precipitation. The material thus prepared is then activated to serum accelerator globulin followed by isolation on a chromatographic column.

Although the products thus prepared are not chemically homogeneous, they have the following properties:

1) An ultraviolet spectrum similar to a protein-peptide containing an aromatic amino acid.

2) A mobility between that of a beta and gamma globulin as measured by filter paper electrophoresis.

3) Loss of activity upon dialysis which may be due to destruction or adsorption.

4) Marked instability which can be somewhat minimized in the presence of 50 per cent glycerol.

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EXCRETION OF SODIUM AND POTASSIUM IN HUMAN SWEAT [1]

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Most studies of sweat composition have dealt with sweat obtained after a thermal stimulus (1). Such stimulation may influence other organs which in turn may influence the function of the sweat glands. To avoid such secondary effects we have induced a localized flow of sweat by injecting a cholinergic drug into a small area of skin and observed the outflow of water and certain solutes. The techniques employed permitted numerous observations, replication, control of the intensity and localization of the stimulus, and exact quantitation of the response. The studies to be reported below are concerned with the excretion of sodium and potassium in sweat obtained under these conditions. The results are considered in relation to possible functional subdivisions of the process of sweat secretion.

PROCEDURE

Fifteen men and two women served as subjects. Six were normal, 30 to 35 years of age; eleven, 25 to 50 years of age, had essential hypertension uncomplicated by cardiac or renal failure. In none of the subjects was there evident endocrine disease, edema or dehydration. The tests were started at least two hours after a light breakfast. Unless otherwise noted the patients were on standard diets providing 175 mEq. of sodium chloride daily and water *ad libitum* until the start of the test. During the test the subject remained at rest in bed.

In a typical experiment sweating was induced in symmetrical forearm sites by intradermal injection of B-methylacetylcholine hydrochloride (Mecholyl), 2 mg. in 0.5 ml. of isotonic saline. The collection of sweat by a standard procedure (2) has been described previously.

In brief, the unit used for collection of sweat consisted of an aluminum ring cemented to the skin and an aluminum cover assembly containing a rubber compression gasket to insure a gas-tight seal. The chamber formed when the lid was fixed into the ring had an internal diameter of 25 mm. and a height of 1 mm. The sweat was absorbed into one or more filter paper discs, its quantity determined by weight and its content of sodium and potassium by flame photometry using lithium as an internal standard.

In each experiment there were 5 to 20 consecutive collection periods each lasting from 10 to 30 minutes. In all, 53 experiments were completed and 557 samples of sweat collected and analyzed for sodium. Of this group, 362 samples (32 experiments, 9 subjects) were also analyzed for potassium. Most of the collections of sweat were obtained from the proximal medioventral surface of the forearm, while in a few experiments sites on the arms, thighs, abdomen and back were used. During the tests one or more samples of blood were drawn from an antecubital vein, heparinized, centrifuged immediately and the plasma analyzed for sodium and potassium in the same manner as the samples of sweat.

Two experiments served to estimate the precision of the measurements: A solution containing 122 mEq. per L. of sodium and 10 mEq. per L. of potassium was delivered onto filter paper discs in 20 weighing vessels. Each of five vessels received approximately 0.2 ml. of the solution (Na 24 μEq., K 2.0 μEq.); five received 0.1 ml. (Na 12 μEq., K 1.0 μEq.); five received 0.05 ml. (Na 6 μEq., K. 0.5 μEq.); and the remaining five vessels received 0.025 ml. (Na 3 μEq., K 0.25 μEq.). In the second experiment a solution containing 33 mEq. of sodium and 10 mEq. of potassium per liter was delivered onto discs in 15 weighing vessels. Five of the vessels received 0.05 ml. (Na 1.6 μEq., K 0.5 μEq.) five received 0.025 ml. (Na 0.8 μEq., K 0.25 μEq.) and five received 0.125 ml. (Na 0.4 μEq., K 0.13 μEq.). The size of each sample was measured by weight and the quantity of sodium and potassium determined by flame photometry just as in the routine procedure. The results showed that the recovery became less precise with decrease in the absolute quantities of sodium and potassium brought to analysis (Table I). The recoveries of 3 μEq. of sodium and 0.5 μEq. of potassium averaged 101 ± 3.1 per cent and 101.5 ± 5.1 per cent respectively. These are the quantities of sodium and potassium that would be encountered in 50 mg. of sweat containing 60 mEq. per L. of sodium and 10 mEq. per L. of potassium.

RESULTS

Sodium

The concentration of sodium, S_{Na}, rose toward a maximum, S'_{Na}, with increase in the rate of sweating (Figure 1). Over a range of flows from 0.35 to 25.3 gm. per M^2min., S_{Na} varied from 1.9 [2]

[1] This work was presented in part at the Fall Meeting of the American Physiological Society, Madison, Wisconsin, 1954.

[2] This is the lowest significant value found for S_{Na}; but the fact that still lower values prevailed at times was

TABLE I

Recovery of known amounts of sodium and potassium from filter paper discs

Solution	Sodium			Potassium		
	Amount of Na added * $\mu Eq.$	Per cent Mean *	Recovered S.D.	Amount of K added * $\mu Eq.$	Per cent Mean *	Recovered S.D.
A	24.6	99.2	0.2	2.02	100	2.0
	12.6	102	2.0	1.03	101	1.0
	6.11	102	1.8	0.50	105	2.0
	3.12	101	3.1	0.26	116	14.7
B	1.79	95.3	10.0	0.52	98	5.3
	0.84	105	8.0	0.25	104	18.0
	0.42	120	15.0	0.13	123	22.1

* Average of 5 replicates.

to 103.8 mEq. per L., the maximum being well below the concentration in plasma, P_{Na}. In any one experiment, P_{Na} remained constant; in the total of 53 experiments, P_{Na} varied between the extremes of 137 to 153 mEq. per L. without any systematic relation to the concentration in sweat. The ratio, S_{Na}/P_{Na}, averaged 0.39 ± 0.20 with a range from 0.05 to 0.64.

The rate of excretion of sodium in the sweat, E_{Na}, a product of both the concentration, S_{Na}, and the flow, V, rose sharply because of increase in both factors. Therefore at high rates of flow the excretion rate approached a linear function of flow, $E_{Na} = S'_{Na}\ V - T_{Na}$, where S'_{Na} and T_{Na} are constants (Figure 2).

S'_{Na} and T_{Na} were estimated from the linear regressions of E_{Na} on V for values of V above 5 gm. per M^2min., omitting the first period which proved to be atypical (*vide infra*). Values for S'_{Na} and T_{Na} derived from 25 experiments on seven subjects are given in Table II. S'_{Na} averaged 56.5 ± 29.1 mEq. per L. with a range from 7 to 96 mEq. per L.; T_{Na} averaged 185 ± 100 μEq. per M^2min. with a range from 19 to 300 μEq. per M^2min. S'_{Na} and T_{Na} were found to vary greatly from subject to subject but very much less in replicate studies involving the same subject (Table II). The variance of S'_{Na} and T_{Na} from site to site in the same experiment was significantly less ($p <$

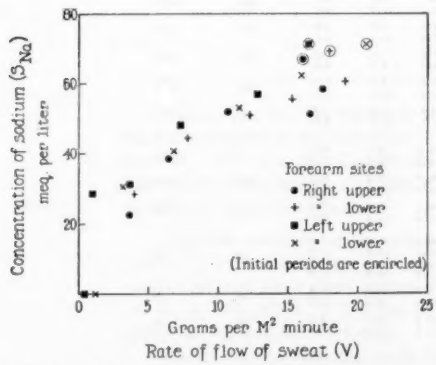

FIG. 1. RELATION BETWEEN THE CONCENTRATION OF SODIUM IN SWEAT AND THE SWEATING RATE

The data presented are from a typical experiment, each point representing an individual collection period. An encircled point refers only to data from the first collection period at each test site.

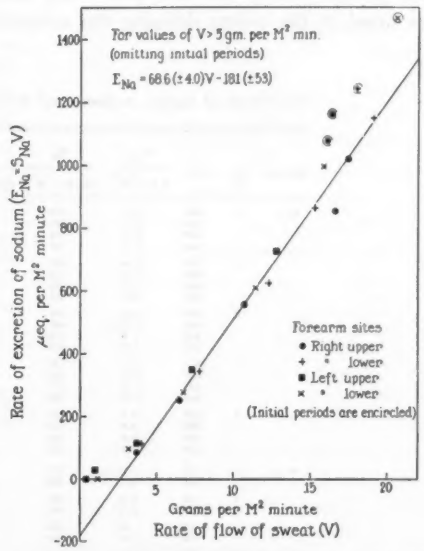
For values of V > 5 gm. per M² min. (omitting initial periods)
E_{Na} = 68.6 (±4.0) V - 181 (±53)
Rate of excretion of sodium (E_{Na} = S_{Na} V) μeq. per M² minute
Forearm sites
Right upper
lower
Left upper
lower
(Initial periods are encircled)
Grams per M² minute
Rate of flow of sweat (V)

FIG. 2. RELATION BETWEEN THE RATE OF EXCRETION OF SODIUM IN THE SWEAT AND THE SWEATING RATE

The data are calculated from the experiment shown in Figure 1. An encircled point refers only to data from the first collection period at each test site.

evident from the occasional failure of our analytical method to detect any sodium in sweat samples obtained at flow rates of 2 gm. per M²min. or less.

0.01) than the variance from experiment to experiment at the same site, indicating that the total variance of these parameters was due chiefly to physiological factors and not to experimental technique.

The concentration of sodium in the sweat of the collection period immediately following the stimulating injection was not related to the sweating rate in the same manner as the concentration of sodium in the sweat of the subsequent collection periods. The point defining the concentration of sodium and the sweating rate of the initial collection period usually fell well above any simple curve fitted to the points defining the concentrations of sodium and the rates of flow of sweat of the subsequent periods (Figure 1). In several experiments the sweating rate during the second collection period was the same as or greater than the sweating rate during the initial period; in each of these experiments the concentration of sodium in the initial sample of sweat was invariably higher than the concentration of sodium in the sample of sweat obtained in the following period. This difference proved to be significant ($p < 0.01$).

Potassium

In general the concentration of potassium in the sweat, S_k, remained constant as the rate of

TABLE II

Excretion of water, sodium and potassium in sweat after intradermal Mecholyl †*

Patient	Exp. No.	Site	Q_{H_2O} g $min^{-1}m^{-2}$	Q_{Na} meq $min^{-1}m^{-2}$	S_k meq $min^{-1}m^{-2}$	S_{Na}/Q_{H_2O} meq L^{-1}	S_k/Q_{H_2O} meq L^{-1}	S_{Na}/S_k	S'_{Na} meq L^{-1}	T_{Na} meq $min^{-1}m^{-2}$	
M.W.	1	RUF	13.3	774	87.4	59	6.6	8.9	75	-380	
		RLF	13.7	836	91.0	60	6.7	9.1	77	-370	-300
		LUF	11.4	740	75.3	65	6.6	9.9	82	-310	
		LLF	13.4	795	80.6	49	5.0	9.9	86	-340	
	2	RUF	11.9	639	78.7	54	6.6	8.1	68	-210	
		RLF	12.8	724	82.5	57	6.5	8.8	71	-230	-238
		LUF	6.9	397	46.1	57	6.6	8.7	74	-220	
		LLF	9.9	573	69.1	58	7.0	8.3	79	-290	
	3	RUF	9.2	356	76.3	39	8.5	4.6	59	-220	
		RLF	11.0	487	89.8	44	8.1	4.9	61	-330	-233
		LUF	7.5	312	65.5	42	8.7	4.8	63	-240	
		LLF	6.8	286	61.4	43	9.1	4.7	61	-240	
	4	RUF	13.0	767	88.0	59	6.8	8.7	79	-260	
		RLF	13.0	763	83.8	64	7.0	9.1	75	-170	-255
		LUF	10.5	617	77.2	59	7.4	8.0	80	-230	
		LLF	11.9	730	86.6	61	7.3	8.4	83	-360	
	5	RUF	10.4	756	67.3	73	6.5	11.2	88	-180	
		RLF	6.7	728	53.6	64	8.2	13.6	93	-190	-190
		LUF	8.6	630	61.8	73	7.2	10.2	103	-200	
		LLF	9.7	766	66.1	80	7.1	11.3	99	-200	
M.L.	1	RF	7.2	318	69.6	44.2	9.7	4.6	78	-270	
		LF	8.7	398	86.2	46.0	10.0	4.6			
	2	RF	8.2	387	79.0	47.0	9.6	4.9	77	-300	
		LF	8.2	391	83.4	47.8	10.2	4.7			
	3	RF	7.0	360	70.0	51.3	9.9	5.2	65	-140	
		LF	10.6	595	102.4	55.4	9.5	5.8			
	4	RF	6.1	238	57.6	37.5	9.5	4.0	69	-260	
		LF	8.1	288	81.0	36.6	10.0	3.7			
	5	RF	10.8	522	96.2	49.5	9.1	5.4	72	-200	
		LF	7.8	367	80.4	47.3	10.4	4.6			
	6	RF	6.1	266	63.4	44.0	10.5	4.2	79	-310	
		LF	5.8	250	50.6	43.4	9.8	5.0			
	7	RF	6.7	369	60.0	54.7	8.9	6.2	81	-270	
		LF	6.5	302	66.2	46.2	10.1	4.6			
	8	RF	5.1	252	49.0	49.6	9.5	5.3	77	-190	
		LF	5.5	280	60.8	51.0	11.2	4.6			
L.F	1	RUF	7.1	77	74.0	10.9	10.4	1.0			
		RLF	8.6	66	69.0	11.4	11.9	1.0	19	-72	
		LUF	7.7	96	83.4	12.5	10.7	1.2			
		LLF	7.1	80	77.0	11.3	10.9	1.0			
	2	RF	7.9	107	89.0	13.7	11.3	1.2	31	-84	
		LF	7.3	90.4	76.4	13.4	10.5	1.2			
	3	RF	5.5	84.6	69.8	15.4	12.7	1.2	20	-45	
		LF	6.4	97.6	73.0	15.3	11.4	1.3			
	4	RA									
		LA									
	5	LF	10.0	155	87.6	15.6	8.8	1.8			
F McC.	1	RF	13.9	134	148	9.9	10.5	0.9	9	-30	
		LF-	12.4	122	129	9.9	10.4	1.0			
	2	RF	12.8	93	132	7.3	10.3	0.7	7	-19	
		LF	11.7	73	106	6.2	9.0	0.7			
	3	RF	11.6	88	122	7.6	10.5	0.7	7	-37	
		LF	12.1	75	118	6.2	9.7	0.6			
	4	RF	6.9	75	77	10.9	11.2	1.0	13	-43	
		LF	6.6	70	67	10.7	9.5	1.1			
J.T.	1	RF	10.0	381	104	38.1	10.4	3.7	60	-230	
	2	RF	7.6	256		34.0					
		LF	6.6	396		60.5					
	3	RF	11.0	473		43.0			57	-280	
		LF	10.7	463		43.2					

TABLE II.—*Continued*

Patient	Exp. No.	Site	Q_{H_2O} gm $min^{-1}M^{-2}$	Q_{Na} meq $min^{-1}M^{-2}$	Q_K meq $min^{-1}M^{-2}$	Q_{Na}/Q_{H_2O} meq L^{-1}	Q_K/Q_{H_2O} meq L^{-1}	Q_{Na}/Q_K	S'_{Na} meq L^{-1}	T_{Na} meq $min^{-1}M^{-2}$
I.B.	1	RTh	3.0	169		56.3				
		LTh	3.0	165		55.0				
	2	RTh	3.4	261		77.2				
		LTh	3.7	187		69.4				
	3	RTh	4.1	226		55.7				
		LTh	3.4	198		57.9				
	4	Th(R+L)	3.8	149	36.8	39.1	9.4	4.3		
		F(R+L)	7.3	294	66.3	40.5	9.1	4.4		
	5	RTh	6.7	210	64.4	31.4	9.5	3.3		
		LTh	5.2	140	47.5	26.7	9.2	3.1		
	6	RF	5.1	211	44.6	41.5	8.8	4.7		
		LF	5.4	248	49.7	45.7	9.2	5.0		
I.B.	1	RF	4.5	166	38.7	37.7	8.7	4.4		
		LF	3.7	130	31.8	34.5	8.7	4.0		
J. D'A.	1	RF	3.4	91		27.1				
		LF	4.8	97		20.1				
	2	LF	5.4	168		31.2				
	3	RF	4.2	205		49.2				
		LF	4.1	186		46.0				
	4	RF	6.0	331		56.5			71	-180
		LF	5.8	293		50.8				
	5	RF	6.2	262		42.6			50	-140
		LF	6.0	271		45.3				
C.S.	1	RF	4.0	238		60.4			89	-250
		LF	7.9	471		59.4				
	2	RF	4.6	196		43.6				
		LF	4.6	179		38.5				
L.D.	1	RF	3.0	247		85.2				
		LF	3.5	294		85.0				
J.S.	1	LB	4.4	135		30.4				
	2	RF	5.5	182		33.4				
	3	RF	7.1	243		34.0				
	4	RF	3.2	90		28.0				
	5	LF	4.6	135		29.7				
A.N.	1	LF	5.1	263		52.0				
N.F.	1	RF	5.1	167	40.4	33.0	8.0	4.2		
		LF	4.1	140	33.0	34.1	8.0	4.2		
W.P.	1	RF	2.2	93		40.6				
		LF	3.0	99		33.4				
A.Y.	1	RF	2.8	65		23.0				
		LF	3.5	97		27.3				
	4	RF	1.6	64		39.8				
		LF	2.6	104		39.3				
	3	RF	1.1	19		17.1				
		LF	1.8	55		30.7				
G.C.	1	RF	2.1	118		56.0				
		LF	2.0	116		56.8				
V.P.	1	RF	1.5	22	29.6	11.4	20.3	0.6		
		LF	1.8	20	40.2	19.2	21.6	0.5		

* Q_{H_2O}, Q_{Na} and Q_K represent total quantities obtained in the serial periods at each site divided by the collection time in minutes and corrected to a standard skin area of 1 M^2. S'_{Na} and T_{Na} are the slope and intercept, respectively, of the linear regression of the rate of excretion of sodium on the rate of flow of sweat for flows exceeding 5 gm. per M^2min.

† Sodium chloride intake was unrestricted but not administered quantitatively to the six normal subjects: J. T., I. S., I. B., L. D., A. N., and G. C. The hypertensive patients were given sodium chloride in the following amounts daily: M. H., M. L., J. D'A., C. S., J. S., and N. F., 175 mEq.; L. F., F. McC., and M. F., 22 mEq.; A. Y., and W. P., 5 mEq.

Values for S_{Na} and T_{Na} could be estimated only for the experiments in which the rate of flow of sweat exceeded 5 gm. per M^2min. during at least 3 of the collection periods. In subject, M. H., the high sweating rate permitted estimation of S'_{Na} and T_{Na} for each site as well as the average values for each experiment.

‡ RF—right forearm, LF—left forearm, RUF—right upper forearm, RLF—right lower forearm, LUF—left upper forearm, LLF—left lower forearm, A—arm, Th—thigh, B—back.

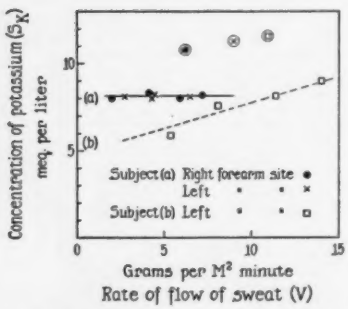

FIG. 3. RELATION BETWEEN THE CONCENTRATION OF POTASSIUM IN THE SWEAT AND THE SWEATING RATE

(a) Illustrates the relation that is usually observed; (b) illustrates the atypical response. An encircled point refers only to data from the first collection period at each test site.

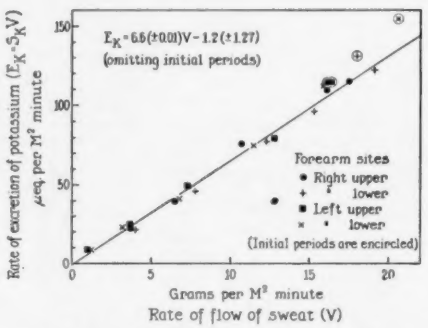

FIG. 5. RELATION BETWEEN THE RATE OF EXCRETION OF POTASSIUM IN THE SWEAT AND THE SWEATING RATE

Data are from the same experiment as the data of Figures 1 and 2. An encircled point refers only to data from the first collection period at each test site.

sweating, V, varied from 0.35 to 25.3 gm. M²min. (Figure 3a); therefore, the rate of excretion of potassium in the sweat, E_k, a product of the concentration and flow, increased in linear proportion to V throughout the entire range of V (Figures 4 and 5). Two of the experiments proved to be atypical in that S_k, like S_{Na}, rose with increase in V (Figure 3b), but, unlike S_{Na}, did not appear to approach a maximal value.

S_k was proportional to the concentration of potassium in plasma, P_k, which remained constant in any one experiment but ranged from 3.7 to 5.6 mEq. per L. in the total of 32 experiments. The ratio of the mean concentration of potassium in the sweat to the concentration in plasma, $\overline{S_k}/P_k$, averaged 2.2 ± 0.48 with a range from 1.7 to 4.2.

The samples of sweat obtained from the period immediately following the injection of Mecholyl had a higher concentration of potassium than the subsequent periods. The concentration of potassium in the sweat of 68 initial collection periods, $\overline{S_K^0}$, averaged 10.7 ± 2.9 mEq. per L.; excluding the data from the initial periods, the sweat potassium concentration averaged 8.9 ± 2.5 mEq. per L. This difference proved to be significant ($p < 0.01$).

The ratio of the potassium concentration in the initial sweat samples to the concentration in plasma, S_K^0/P_K, averaged 2.5 ± 0.27 with a range from 1.9 to 4.4. In the experiments in which 4 sites were stimulated simultaneously, the sweating rate during the initial period, Vo, varied enough from site to site to show that S_K^0 was independent of Vo.

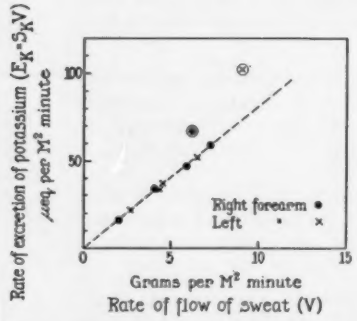

FIG. 4. RELATION BETWEEN THE RATE OF EXCRETION OF POTASSIUM IN THE SWEAT AND THE SWEATING RATE

The data are calculated from the experiment shown in Figure 3a. An encircled point refers only to data from the first collection period at each test site.

Summation of water, sodium and potassium excretion during the entire response to stimulation

The total quantities of water, Q_{H_2O}, sodium, Q_{Na}, and potassium, Q_K, excreted from each site during a test were determined by adding the amounts of water, sodium and potassium obtained in the successive periods. If all the sweat following a single injection of Mecholyl were collected as a single sample, the concentration of sodium and potassium would be Q_{Na}/Q_{H_2O} and Q_K/Q_{H_2O}, respectively. The value of Q_{Na}/Q_{H_2O} ranged from 6.2 to 85.2 mEq. per liter, averaging 39.5 ± 18.4 mEq. per L. (Table II); Q_K/Q_{H_2O} averaged 9.4 ± 2.5 mEq. per L. with a range from 5.0 to 21.8 mEq. per L. (Table II). Both Q_{Na}/H_2O

and Q_K/H_2O varied greatly from subject to subject, but very much less variation was evident in replicate experiments carried out on the same subject (Table II). In addition the variance of these parameters from site to site in the same experiment was significantly less ($p < 0.01$) than from experiment to experiment at the same site, indicating that the total variance of both Q_{Na}/Q_{H_2O} and Q_K/Q_{H_2O} was due predominantly to physiological and not technical factors.

The ratio of the total excretion of sodium to the total excretion of potassium, Q_{Na}/Q_K, averaged 4.3 ± 3.4 with a range from 0.64 to 13.6. In the initial period following stimulation the ratio of the excretion of sodium to the excretion of potassium, S_{Na}^0/S_K^0, averaged 5.5 ± 3.4 with a range from 0.9 to 14.4.

No noteworthy differences were observed when the data obtained from the normal subjects were compared with that obtained from the hypertensive patients on a high salt intake. Nonetheless it is of interest that the factor of hypertensive disease appeared to be dissociated from the factor of sodium chloride intake, since patients W. P. and A. Y., in whom the sodium chloride intake was most severely restricted (5 mEq. daily), were not found to have the lowest concentrations of sodium in the sweat. In fact, the lowest values of sweat sodium concentration and the highest value of sweat potassium concentration were observed in the hypertensive patients, F. McC., L. F., and M. F., in whom the salt intake was more than four times as great (22 mEq. daily).

DISCUSSION

The lower concentration of sodium in sweat as compared to plasma could occur as a result of primary movement of water by the tubular cells from the extracellular fluid to the gland lumen followed by diffusion of sodium ions insufficient to restore concentration equilibrium. If, in this schema, some of the diffusing sodium ions were to be engaged and then transported by another cellular process oriented in the reverse direction, *i.e.*, from the forming sweat to the extracellular fluid, the sodium concentration of the sweat would be further lowered. Alternatively, if both water and sodium were transported across the tubular epithelium in one direction in one portion of the gland and in the opposite direction in another portion of the gland, the lower concentration of sodium in sweat relative to extracellular fluid would be a consequence of a net dominance of water transport lumenwards, and/or sodium transport in the opposite direction. A simpler explanation for the production of sweat of low sodium concentration, namely, reabsorption of sodium from an ultrafiltrate of plasma (as in the kidney), is unacceptable *a priori* because of the lack in the structure of the sweat gland of a filtering bed comparable to the glomerulus. Furthermore it has been shown in physiological studies that the sweat gland presents a barrier to the free diffusion from extracellular fluid to tubule lumen of small molecules that pass through the glomerular filter without hindrance (3).

In a previous paper we have suggested that a precursor solution is formed in variable amounts in the sweat glands, that a constant fraction of this solution is returned to the extracellular fluid, and, therefore, that the sweating rate is proportional to the rate of formation of precursor (2). In the present work it has been shown that the rate of excretion of sodium in the sweat, E_{Na}, is not a linear function of the rate of flow of sweat, nor, therefore, of the rate of formation of precursor. The increment in the rate of excretion of sodium in the sweat, dE_{Na}, rises with increment in the sweating rate, dV, and, therefore, with increment in the rate of formation of precursor. The ratio dE_{Na}/dV appears to approach a maximum limiting value, a phenomenon which is explained if it is assumed that the sweat gland forms a precursor solution of constant sodium concentration from which sodium is reabsorbed by a process of limited capacity. Under this assumption the quantity of sodium presented per unit time to the reabsorbing system, that is, the rate of input of sodium, depends on the concentration of sodium in the precursor and the rate of formation of precursor. At the lowest rates of formation of precursor, the rate of input of sodium will be less than the reabsorptive capacity, and none will appear in the sweat. With increasing rates of formation of precursor the sodium input will equal and then exceed the maximal rate of reabsorption; at this point sodium appears in the sweat collected on the skin surface.[8] With fur-

[8] Since the tubules may be expected to vary in functional capacity as well as size, the glands with the smallest maximal reabsorptive capacity for sodium will per-

ther increase in the rate of formation of precursor the rate of reabsorption of sodium becomes a progressively smaller fraction of the rate of input of sodium and thus the rate of excretion of sodium in the sweat approaches the rate of input asymptotically. The slope of this asymptote, the maximum input of sodium per unit quantity of sweat produced, is identical with the limiting concentration of sodium, S'_{Na}, which is approached at rates of flow exceeding 5 gm. per M^2min.; the intercept, T_{Na}, can be interpreted as the maximal capacity of the glandular mechanism for reabsorption of sodium from a precursor solution (Table II).

This hypothesis has been suggested as an explanation of the relation between the concentration of sodium and the rate of flow of salivary secretion (4). The rate of excretion of sodium in parotid saliva was found to approximate a linear function of the rate of flow of saliva at flows above 1 ml. per minute. A linear regression of the rate of sodium excretion on the rate of salivary flow has a slope, S'_{Na}, of 107 ± 3.8 mEq. per liter and an intercept, T_{Na}, of 60 ± 7.8 μEq. per minute. In view of the fact that the subjects of both the saliva and the sweat studies were maintained in the same environment with similar activities, diets and daily intakes of sodium, it is of interest that the mean value of S'_{Na} for saliva was greater than any single value and approximately twice the mean value of S'_{Na} for sweat. The parameter, T_{Na}, cannot be directly compared, since in the case of saliva, it is influenced by the amount of functioning parotid tissue in the gland studied, and, in the case of the sweat, it is influenced by the dimensions and density of glands in the skin area sampled.

In the case of potassium, a reabsorptive process is not suggested since the rate of excretion, E_K, was found in general to be simply proportional to the sweating rate, the curves passing through the origin (Figures 4 and 5).

SUMMARY

The concentrations of sodium and potassium in human sweat were measured following stimulation of sweating by local injection of acetyl B methylcholine.

The concentration of sodium rose toward a maximum with increase in the sweating rate but was always lower than the concentration in the plasma. The mean concentration of sodium in the total output of sweat from each stimulated site ranged from 6.2 to 85.2 mEq. per L. averaging 39.5 ± 18.4 mEq. per L. The concentration of sodium in the sweat obtained in the initial collection period was invariably higher than the concentration of sodium in the sweat collected in any subsequent period even when the sweating rate was lower in the initial than in the subsequent period.

The concentration of potassium in the sweat, S_K, was greater than the concentration in the plasma, P_K, the ratio S_K/P_K averaging 2.2 ± 0.48. In general S_K remained constant as the sweating rate declined; exceptionally S_K decreased with decrease in the sweating rate. The average concentration of potassium in the sweat obtained in the initial collection periods was significantly higher ($P < 0.01$) than the average concentration of potassium in the sweat collected subsequently. The concentration of potassium in the total output of sweat from each stimulated site ranged from 5.0 to 21.8 mEq. per L., averaging 9.4 ± 2.5 mEq. per L.

The relation of the rate of excretion of sodium and potassium to the rate of flow of sweat is compatible with the hypothesis that both ions are delivered into a precursor solution and that sodium, but not potassium, is reabsorbed by a subsequent process of limited capacity.

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mit the appearance of sodium excretion at the lowest rates of flow. This would explain the appearance of sodium in the sweat at times when the average rate of input of sodium into the glands is less than the average reabsorptive capacity.

THE EFFECT OF PLASMA ON THE *IN VITRO* UPTAKE OR BINDING BY HUMAN RED CELLS OF RADIOACTIVE I–131 LABELED L–THYROXINE AND L–TRIIODOTHYRONINE

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Thyroxine is bound to a serum protein which appears in the electrophoretogram just ahead of alpha-2 globulin (1–4). Triiodothyronine also associates with this thyroxine binding globulin (T.B.G.) but can be displaced readily with thyroxine while triiodothyronine will not readily displace thyroxine. Deiss, Albright, and Larson (5) suggested that the firm binding of thyroxine to T.B.G. might account for the slower rate of disappearance of thyroxine from the plasma as compared to triiodothyronine.

The purpose of this paper is to report studies on the ***in vitro*** uptake or binding by human red cells of radioactive I-131 labeled sodium iodide, human serum albumin, triiodothyronine, and thyroxine. We propose to show that the binding of L-thyroxine by plasma is one of the limiting factors in the uptake or binding of this substance by the human red cell. The uptake or binding of L-triiodothyronine is limited to a lesser degree by plasma binding and is consistently greater than the uptake or binding of L-thyroxine.

MATERIALS AND METHODS

Blood was obtained from healthy human volunteers in heparinized syringes and the cells separated from the plasma by centrifugation. Stock solutions of radioactive (I-131) labeled sodium iodide, human serum albumin,[1] L-thyroxine,[1] and L-triiodothyronine [1] were diluted with normal saline solution so that two milliliters of the diluted solution contained approximately 2×10^3 counts per minute. Two milliliters of each solution was placed in a test tube and the radioactivity measured in a deep well scintillating counter. Two milliliters of unwashed red blood cells were then added to each of the solutions, mixed well, and incubated in a water bath at 37°C for an arbitrary period of 30 minutes. The tubes were then centrifuged, supernatant removed, the cells were washed and recentrifuged three times. The supernatant removed and the radioactivity remaining with the red cells was determined and was expressed as a percentage uptake of the original radioactivity. The same procedure was repeated using red blood cells which had been washed three times with normal saline before adding them to the radioactive solutions. (These will be referred to as washed cells.)

Radioactive (I-131) labeled sodium iodide, L-thyroxine, and L-triiodothyronine were dissolved in human plasma (obtained from the Blood Bank) and the same procedure described for the saline solution was carried out using both "washed" and "unwashed" red blood cells.

Two milliliters of plasma were incubated in a H_2O bath at 37°C for 30 minutes with 0.1 milliliter of an alkaline solution of stable L-thyroxine containing 4.5 micrograms per milliliter. Varying amounts of this plasma (.02 ml to 0.5 ml) were added to tubes containing 2 milliliters of radioactive (I-131) labeled L-thyroxine in normal saline (previously counted) and 2 milliliters of "washed" red cells were added immediately, mixed gently, and incubated in a water bath at 37°C for 30 minutes. At the end of 30 minutes the tubes were centrifuged, the supernatant removed, and the cells washed three times with normal saline. The amount of radioactivity remaining with the red cells was determined and expressed as described previously. The same procedure was repeated using radioactive L-triiodothyronine instead of radioactive L-thyroxine.

Two milliliters of unwashed red cells were incubated at 37°C for 30 minutes with 50, 100, and 150 micrograms of stable L-thyroxine dissolved in 0.1 ml. of .02 N sodium hydroxide. The above mixture was then incubated with radioactive I-131 labeled L-thyroxine for 30 minutes at 37°C. The amount of radioactivity remaining with the red cells was determined and expressed as described previously. The same procedure was repeated using 100 micrograms of stable L-triiodothyronine [2] instead of L-thyroxine.

In all of the experiments where an alkaline solution

[1] Obtained from Abbott Laboratories, North Chicago, Ill. The radioactive human serum albumin (RISA) was kindly furnished through the courtesy of Dr. Donalee S. Tabern. Two milliliters of the diluted solutions contained approximately 2.5×10^{-4} micrograms of L-thyroxine or L-triiodothyronine.

[2] Kindly furnished by Dr. Arthur Heming of Smith, Kline, and French Laboratories, Philadelphia, Pennsylvania.

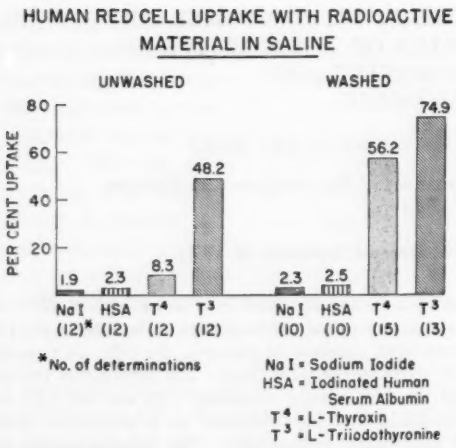

FIG. 1. A COMPARISON OF THE PERCENTAGE UPTAKE OF WASHED VERSUS UNWASHED RED CELLS WHEN THE LABELED MATERIALS WERE DISSOLVED IN NORMAL SALINE

of stable L-thyroxine was used a control was set up using the alkaline solution without thyroxine. The radioactivity remaining with the red cell was not due to absorption of the radioactive L-thyroxine or L-triiodothyronine as less than one per cent of the activity will adhere to the glass test tube after saline washings.

RESULTS

Unwashed red cells—Radioactive tracers in normal saline solution

The percentage uptake by or the percentage of radioactivity remaining with the unwashed red cells was negligible with both radioactive I-131 labeled sodium iodide and human serum albumin. The uptake of labeled L-thyroxine was considerably increased (mean 8.3 per cent) over that of labeled sodium iodide (mean 1.9 per cent) and human serum albumin (mean 2.3 per cent). The uptake of labeled L-triiodothyronine was markedly increased (mean 48.2 per cent) above that for L-thyroxine (mean 8.3 per cent) (Figure 1).

Washed red cells—Radioactive tracers in normal saline solution

The percentage uptake by the washed red cells of the labeled sodium iodide and human serum albumin was again negligible. The uptake of labeled L-thyroxine (mean 56.2 per cent) was markedly increased over that of sodium iodide (mean 2.3 per cent) and human serum albumin (2.5 per cent). This was also a marked increase over that of the uptake of L-thyroxine (mean 8.3 per cent) by the unwashed red cells. The percentage uptake of labeled L-triiodothyronine (mean 74.9 per cent) was increased above that of L-thyroxine and above the percentage uptake in the unwashed cells (mean 48.2 per cent) (Figure 1).

Washed and unwashed red cells—Radioactive tracers in human plasma

The percentage uptake of the labeled sodium iodide, L-thyroxine, and L-triiodothyronine in plasma by the unwashed red cell was 2.6, 7.1, and 15.0 per cent, respectively. The main difference as compared with radioactive tracers in saline was with L-triiodothyronine, *e.g.*, 48.2 per cent uptake in saline and 15.0 per cent uptake in plasma.

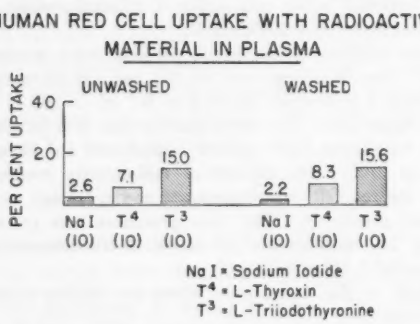

FIG. 2. A COMPARISON OF THE PERCENTAGE UPTAKE OF WASHED VERSUS UNWASHED RED CELLS WHEN THE LABELED MATERIALS WERE DISSOLVED IN HUMAN PLASMA

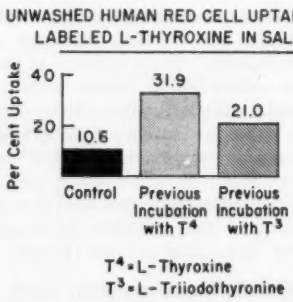

FIGURE 3.

Previous incubation of the unwashed red cell with stable L-thyroxine markedly increases the uptake of the labeled L-thyroxine. L-triiodothyronine incubation increases it to a lesser degree.

When washed red cells were used, the percentage uptake of the labeled sodium iodide, L-thyroxine, and L-triiodothyronine being 2.2, 8.3, and 15.6 per cent, respectively, did not differ significantly from those obtained with unwashed cells. However, there was a marked difference in the uptake of L-thyroxine and L-triiodothyronine as compared to the uptake by washed red cells when the substances were in normal saline. The uptake of L-thyroxine dropped from 56.2 per cent to 8.3 per cent and L-triiodothyronine from 74.9 per cent to 15 per cent (Figure 2).

Effect of incubating unwashed red cells with stable L-thyroxine and L-triiodothyronine on the uptake of labeled L-thyroxine

The percentage uptake by unwashed red cells of labeled L-thyroxine was definitely increased by previous incubation of the cells with stable L-thyroxine (10.6 per cent to 31.9 per cent). It was increased to a lesser degree by previous incubation of the cells with stable L-triiodothyronine (10.6 per cent to 21.0 per cent) (Figure 3).

Effect of adding plasma on the percentage uptake of labeled L-thyroxine by washed red cells

When varying amounts of plasma, varying from .02 to 0.5 ml, were added back to washed red cells there was a definite decrease in the percentage uptake of labeled L-thyroxine in saline, *i.e.*, 55 per cent to 10 per cent (Figure 4). The plasma was incubated with stable L-thyroxine before adding it back to the washed red cells. It was impossible to add small enough quantities of plasma to demonstrate a concentration curve unless it was previously incubated with stable L-thyroxine. The uptake of radioactive L-triiodothyronine was also inhibited by the presence of plasma decreasing from 76 per cent to 24 per cent (Figure 4).

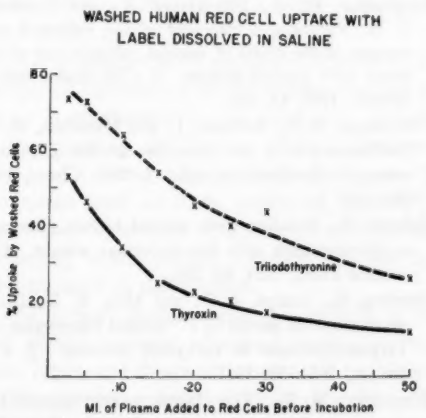

FIG. 4. A CURVE SHOWING THAT INCREASING AMOUNTS OF PLASMA ADDED BACK TO WASHED RED CELLS CAUSES A MARKED DECREASE IN THE UPTAKE OF LABELED L-THYROXINE AND L-TRIIODOTHYRONINE

DISCUSSION

The possibility that the uptake or binding [3] by the red cell of L-thyroxine or L-triiodothyronine is due to contamination with inorganic iodide or a non-specific organic iodinated compound is eliminated by the insignificant uptake by the red cells of radioactive I-131 labeled sodium iodide and iodinated human serum albumin.

Studies from several laboratories (1–5, 6) have consistently shown that thyroxine is bound to a serum protein which appears in the electrophoretogram just ahead of alpha-2 globulin (inter-alpha zone). Petermann, Robbins, and Hamilton (7) have further characterized this protein and Schmid (8) found the greatest concentration of protein bound iodine to be in an alpha-glycoprotein fraction.

The studies reported here suggest that in at least one cell, the human erythrocyte, the binding of thyroxine by serum protein is a limiting factor in the uptake [4] of radioactive I-131 labeled L-thyroxine. The uptake by the unwashed red cell of L-thyroxine dissolved in saline and by the washed red cell of L-thyroxine dissolved in plasma was markedly decreased compared to the uptake by washed red cells of L-thyroxine dissolved in saline. This decreased uptake due to plasma was further demonstrated by the decreasing uptake as increased amounts of plasma were added back to the washed red cells before incubation with the labeled L-thyroxine (Figure 4).

The uptake by the unwashed red cell of labeled L-triiodothyronine was considerably greater than that of the labeled L-thyroxine (Figure 1).

[3] To avoid confusion this will be referred to as uptake.

[4] It should be emphasized again that by uptake or binding we mean radioactivity that remains with the red blood cell after repeated saline washings. This radioactivity could be within the cell or on the surface of the cell membrane.

This was also true of the uptake by the washed cells although the difference was considerably reduced due to the marked increase in uptake by the washed red cell of L-thyroxine. The uptake by both washed and unwashed red cells of labeled L-triiodothyronine dissolved in plasma was markedly reduced compared to the saline solution. The above studies suggest that plasma will bind L-triiodothyronine but to a lesser degree than L-thyroxine. This may be due to the triiodothyronine-protein binding which is less specific than thyroxine since triiodothyronine is found in all the electrophoretically distinguishable protein bands (6). The decreased binding of L-triiodothyronine compared to L-thyroxine was also demonstrated by previous incubation of unwashed red cells with stable L-triiodothyronine and L-thyroxine in saline before incubation with labeled L-thyroxine. The uptake was definitely increased by incubation with L-thyroxine but to a lesser degree with L-triiodothyronine (Figure 3).

These studies tend to confirm the binding of L-thyroxine by plasma and to show that the *in vitro* uptake of labeled L-thyroxine by the red cell is markedly decreased in the presence of plasma. The uptake of L-triiodothyronine is also decreased but to a lesser degree. This could be a factor in the more rapid disappearance of L-triiodothyronine from the serum of euthyroid subjects (9). It should be mentioned that the difference in uptake could be due to a greater affinity of the red cells for L-triiodothyronine than for L-thyroxine. These studies do not demonstrate which interpretation is correct.

Hamolsky (10) has presented evidence that there is a qualitative difference in the plasma protein-thyroid hormone complex in the patient with diffuse toxic goitre versus the euthyroid state. This difference was demonstrated by an increased uptake by normal red cells of radioactive I-131 labeled plasma from patients with toxic diffuse goitre. We are attempting to confirm this observation by the methods used in this study. It would appear that this difference is probably due to binding as Dingledine, Pitt-Rivers, and Stanbury (6) were unable to demonstrate any difference in the iodinated compounds of the blood in euthyroid versus hyperthyroid subjects.

SUMMARY

1. The uptake or binding by the human red blood cell of radioactive I-131 labeled triiodothyronine was consistently greater than the uptake of radioactive I-131 labeled thyroxine.

2. The presence of plasma markedly decreased the uptake or binding by the human red cell of radioactive I-131 labeled thyroxine and to a lesser degree the uptake or binding of radioactive I-131 labeled triiodothyronine.

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A RELATION BETWEEN HEMODYNAMIC AND PLASMA VOLUME ALTERATIONS DURING GENERAL ANESTHESIA IN MAN [1]

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Measurements made in man during surgical operations and ether anesthesia have shown that a reduction in blood volume occurs which is too great to be explained by external blood loss (1, 2). On the basis of plasma volume and thiocyanate space estimations, it appears likely that the unexplained part of the change in blood volume results from a loss of plasma water to extracellular fluid (2).

Among the possible causes for such a fluid shift, the most likely is a disparity between capillary filtration and reabsorption, and lymphatic return, produced in turn by changes in the level of blood pressure. The hemodynamic alterations produced by general anesthesia may therefore be of prime importance in determining the net loss or gain of plasma water from the capillary blood. However, hemodynamic measurements have usually not been made during anesthesia in conjunction with blood volume determinations. Further, it has not been sufficiently appreciated that different anesthetic agents produce characteristic circulatory changes which could explain differences in their effects on the plasma volume.

Animal studies have suggested that part of the change in circulating blood volume during anesthesia is attributable to the release or storage of erythrocytes by the spleen (3–6). Changes in the blood volume during anesthesia in man have sometimes been similarly explained, although the importance of splenic dilatation or contraction in producing blood volume changes has yet to be demonstrated in this species.

The present study reports the changes in blood and plasma volume observed during cyclopropane, ether, and thiopental anesthesia in thirty-one healthy patients scheduled for elective operations. Blood volume determinations were made using the dye T-1824 or the isotope Cr^{51}; in addition, arterial and peripheral venous pressures were continuously measured before, during, and after anesthesia and operation.

PROCEDURE

Thirty-one hospital patients scheduled for minor elective operations were studied. The age range was from 23 to 76 years. Different anesthetics were alternated to reduce the influence of seasonal changes.

Procedure A. In ten subjects blood samples were withdrawn into oxalated tubes from the brachial artery before and after the intravenous injection of 15 to 20 mg. T-1824. The disappearance rate of dye from the plasma was estimated from the plasma concentration in blood samples drawn from the brachial artery ten, twenty, thirty, and forty minutes following the injection of dye. The optical densities of the plasma samples were plotted against time on semilogarithmic graph paper and extrapolated to determine the changes in plasma volume occurring after drug administration. Hematocrits were read in quadruplicate on all blood samples and the plasma volume changes were calculated from these measurements as well.

Procedure B. In twenty-one subjects blood was withdrawn from four to fourteen hours prior to the scheduled time of operation and labelled with Cr^{51} according to the method of Gray and Sterling (7). The suspension of tagged cells was injected intravenously into the subject at least one-half hour before the study began. The period of study was from two hours pre-operatively until one or two hours post-operatively.

Pre-anesthetic sedation consisted either of atropine sulfate or scopolamine hydrobromide (0.4 to 0.6 mg. I.V.) in five subjects, while in the remaining sixteen cases morphine sulfate (10 mg. I.V.) was later given.

Anesthesia was induced and maintaind with cyclopropane-oxygen in ten subjects, using a closed system with carbon dioxide absorption. Ether anesthesia was induced with a mixture of nitrous oxide, oxygen and ether and maintained with oxygen-ether in five subjects. In the remaining cases thiopental sodium, supplemented with nitrous oxide-oxygen provided anesthesia. The period between the induction of anesthesia and the beginning of operation ranged from forty-five to eighty minutes.

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METHODS

Blood samples were drawn from the brachial artery. Those for gas analysis were collected in syringes moistened with heparin; those for counting into oxalated tubes. The blood loss from sampling and operation was approximately one hundred milliliters. This loss was not replaced. Correction for the radioactivity lost by hemorrhage affected the results negligibly.

For the determination of plasma T-1824 concentration the optical density of plasma was read at 622.5 mμ against blank plasma. Hematocrits were determined in capped Wintrobe tubes spun at 2300 g. (at the tip) for thirty minutes. The factor 0.98 was applied to correct for trapped plasma (8). Hemoglobin concentration was calculated from the optical density of oxyhemoglobin at 540 mμ. The ratio between counts per minute and hemoglobin concentration was constant within the limits of experimental error in all samples drawn from the same subject; this was also true of the ratio between counts per minute and the peripheral hematocrit. The standard deviation of duplicate counts was ± 0.51 per cent.

The pH of blood samples was determined in a glass electrode at room temperature and corrected to 37°C (9). Nitrous oxide and cyclopropane in blood were determined manometrically by the method of Orcutt and Waters (10), diethyl ether by a modification of the method of Shaffer and Ronzoni (11), and thiopental by the method of Brodie and his co-workers (12). Oxygen and carbon dioxide were determined in a van Slyke manometric gas analysis apparatus, and the pCO_2 was read from a nomogram (13). Samples containing diethyl ether were analyzed for carbon dioxide using the modification suggested by Austin (14).

The red cell volume was calculated as RCV = (total counts injected/counts per ml. of blood after circulatory mixing) × corrected peripheral hematocrit. Total blood volume could be estimated as $BV = \frac{RCV}{0.9 \times Hct.}$, utilizing the value for the whole body-peripheral hematocrit ratio determined by Gibson, Peacock, Seligman, and Sack (15), Chaplin, Mollison, and Vetter (16), and Strumia and his co-workers (17). Similarly, plasma volume change was calculated from the peripheral hematocrit using the formula $PV_t/PV_0 = \left(\frac{1}{0.9\,H_t} - 1\right) \Big/ \left(\frac{1}{0.9\,H_0} - 1\right)$, where the subscripts refer to the quantities measured at different times. The ratio of plasma volumes calculated by dye dilution is $PV_t/PV_0 = OD_0/OD_t$, where OD_0 is the optical density expected (from the extrapolated line) at time t, and OD_t is the value observed at this time.

Arterial pressure was measured from the brachial artery, and venous pressure from an antecubital vein using Lilly capacitance manometers.

RESULTS

I. *Comparison of plasma volume changes measured with tagged plasma protein and erythrocytes*

The calculation of plasma volume changes from the dilution of tagged red cells requires the assumption that the ratio of whole body to peripheral hematocrit remains constant under the condition studied. It is not known whether anesthesia alters this ratio in man; therefore it was considered advisable to compare the plasma volume changes determined simultaneously from the peripheral hematocrit and the plasma protein concentration measured with T-1824.

The plasma volume changes calculated by the two methods agreed satisfactorily up to eighty or ninety minutes after the injection of T-1824, and the values obtained during this interval in ten subjects have been summarized in Table I. After approximately ninety minutes the plasma volume changes measured by dye dilution usually exceeded those calculated from the hematocrit whenever the plasma volume was diminished during anesthesia; the converse was generally true when the plasma volume increased.

II. *Plasma volume changes as related to those in arterial and venous pressures during anesthesia and operation*

Changes in plasma volume and arterial and venous pressures occurring after pre-anesthetic se-

TABLE I

Plasma volume changes associated with the administration of morphine and anesthetic agents

Pre-anesthetic medication or anesthetic agent	Change in plasma volume (per cent)				Difference ± S. E.	Number of observations
	Calculated from hct.		From T-1824 concentration			
	Average	Range	Average	Range		
Morphine	+ 4.8	0 to +16.0	+ 5.4	+0.5 to +21.2	−0.6 ± 1.24	5
Cyclopropane	−10.5	−5.2 to −18.4	−11.8	−5.1 to −24.6	+1.3 ± 1.25	10
Diethyl ether	− 5.8	−2.8 to −7.7	− 6.8	−4.2 to −9.3	+1.0 ± 0.55	6
Thiopental	+ 3.4	1.6+ to +5.0	+ 2.9	0 to +6.1	+0.5 ± 0.95	5

Table II

EFFECTS OF SEDATION AND ANESTHESIA ON PLASMA VOLUME AND ARTERIAL AND VENOUS PRESSURES

Drug	Amt. or Blood Concentration	Average Change in Mean A. P.	Average Change in V. P.	Change in P. V. %	Change in P. V. ml.	Subjects	Observations
Atropine SO_4 or Scopolamine HB_r	0.4-0.6 mg. i. v.	+3mm. Hg**	-------	M +1.7 R -1.1 to +6.8	M +55 R -30 to +210	5	12
As above, plus morphine SO_4	10 mg. i. v.	-4mm. Hg**	-------	M +6.0* R -1.0 to +20.9	M +166 R -30 to +525	16	26
Cyclopropane (light)	6.1-12 mg. %	+3mm. Hg	+7.9 cm. H_2O*	M +1.4 R -6.1 to +11.6	M +40 R -150 to +180	5	14
Cyclopropane (deep)	17 - 26 mg. %	+20mm. Hg*	+14.2 cm. H_2O*	M -8.8* R -6.2 to -17.3	M -312 R -150 to -420	5	11
Diethyl ether (early)	60 - 110 mg. %	+15mm. Hg*	+10.6 cm. H_2O*	M -9.6* R -1.9 to 14.3	M -236 R -60 to -420	5	8
Diethyl ether (late)	84 - 114 mg. %	-1 mm. Hg	+4.2 cm. H_2O	M -4.9* R -3.7 to -7.8	M -121 R -60 to -230	5	8
Thiopental-Nitrous Oxide	0.6 - 2.5) mg. % 23 - 52)	-16 mm. Hg*	-0.3 cm. H_2O	M +3.0* R -2.5 to +7.3	M +108 R -40 to +190	6	16

*Denotes significant change ($p < 0.05$) by Fisher t test
**Auscultatory blood pressure determinations; changes refer to diastolic pressure

Abbreviations: A.P. Arterial pressure, V.P. Venous pressure, P.V. Plasma volume, M. Mean, R. Range.

dation and during anesthesia are summarized in Table II. Each value is an average of the mean of three determinations made at fifteen to twenty-five-minute intervals in each of the patients studied. Values marked with an asterisk represent statistically significant differences from the pre-sedated or pre-anesthetized levels ($p < 0.05$). The changes in plasma volume are calculated sequentially; *i.e.*, the changes calculated after pre-anesthetic sedation use the pre-sedated values as a baseline, while the changes measured during anesthesia refer to the last values obtained before the induction of anesthesia. All blood volume determinations made in the group of twenty-one subjects reported in Table II employed Cr^{51} red cell tagging.

Pre-anesthetic sedation: The administration of atropine or scopolamine had no measurable effect on plasma volume or arterial pressure. Addition of morphine to the pre-anesthetic medication was associated with a significant increase in plasma volume. No consistent change in arterial pressure occurred after morphine was given, although usually there was a decrease. The period of observation varied between forty minutes and one hour and fifteen minutes following the intravenous injection of these drugs.

Cyclopropane: The patients receiving cyclopropane were divided into two equal groups on the basis of the mean concentration of cyclopropane observed in blood samples drawn at fifteen-minute intervals during the period of anesthesia. In the lightly-anesthetized group, adequate ventilation was produced by intermittent compression of the breathing bag of the anesthesia machine synchronous with the patient's inspirations. The pCO_2 in arterial blood averaged 50.0 mm. Hg and O_2 saturation 99 per cent. In the deeply-anesthetized group apnea or severely reduced respiratory exchange was produced by the anesthetic agent, and manually controlled positive pressure respiration was used throughout anesthesia. In

this group the pCO_2 was lower (average 28 mm. Hg: range 18 to 37 mm. Hg). Oxygen saturation averaged 98 per cent.

The first group showed an insignificant change in plasma volume and the arterial pressure was not consistently altered by anesthesia. The venous pressure, however, increased in all cases (average 7.9 cm. H_2O). The second group exhibited a significantly reduced plasma volume with consistently increased arterial and venous pressures (+20 mm. Hg and +14.2 cm. H_2O respectively). The time of these observations ranged between 20 and 73 minutes after the induction of anesthesia; there was no difference, on the average, between the time of observations in the "light" and "deep" groups.

Diethyl ether: Observations on the subjects receiving this anesthetic were divided into two groups—early and late—because most of the patients studied exhibited signs of "excitement" or other undesirable side effects of anesthesia (transient arterial hypertension in four subjects, coughing in one, diminished oxygen saturation of arterial blood in two, and brief struggling in two) during the induction of anesthesia. In the "early" period (17 to 35 minutes after induction) there was a significant decrease in plasma volume, accompanied by consistently increased arterial and venous pressures. The pCO_2 averaged 56 mm. Hg, and oxygen saturation 87 per cent. After the establishment of anesthesia with diethyl ether, all values began to revert toward normal. The plasma volume increased progressively, although it remained significantly reduced in observations made as late as 80 minutes after induction. The changes in arterial pressure were completely reversed and those in venous pressure partially so in the "late" period of anesthesia. Oxygen saturation averaged 97 per cent at this time, and pCO_2 57 mm. Hg.

Thiopental-nitrous oxide: In this group a significant increase in plasma volume occurred, accompanied by a consistent decrease in arterial pressure, but there was no consistent change in venous pressure. The observations were made between 15 and 84 minutes following the induction of anesthesia. Most of the change in plasma volume occurred within the first half-hour. The arterial pCO_2 averaged 46 mm. Hg and oxygen saturation 99 per cent.

Effects of surgical operation: Operation had no consistent effect on plasma volume, arterial pressure, or venous pressure. In some instances a decrease in plasma volume occurred coincident with the beginning of operation; this was always accompanied by an increase in arterial pressure, and

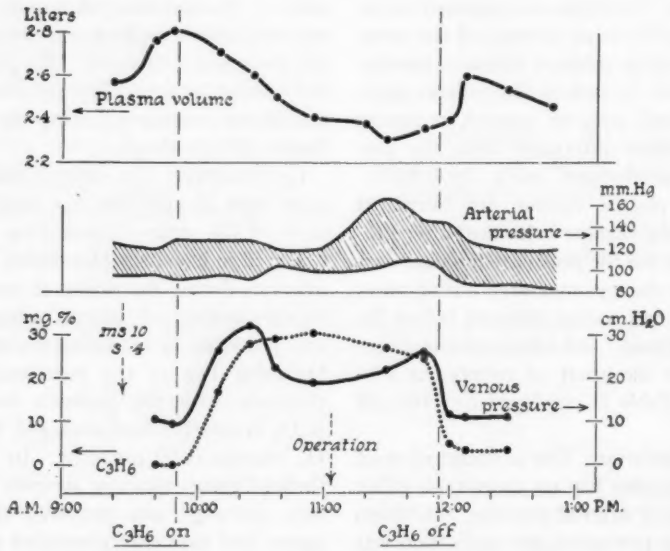

FIGURE 1

usually venous pressure increased also. Figure 1 illustrates a typical example.

Recovery from anesthesia: In all subjects the measured values tended to return toward those observed before the induction of anesthesia. However, plasma volume was usually less upon emergence from anesthesia than just before its induction.

DISCUSSION

The constancy of the relation between counts per minute and the hemoglobin concentration in peripheral blood indicates: 1) complete mixing of tagged and untagged erythrocytes, and 2) lack of an appreciable loss of Cr^{51} from the cells. The first consideration rules out the possibility that sequestered (*i.e.,* unmixed) red cells are added to the circulation during general anesthesia, but does not imply that the distribution of erythrocytes among capillaries, large blood vessels, and "blood reservoirs" such as the spleen is unaffected by anesthetics. However, the agreement between plasma volume changes estimated from simultaneous measurements of peripheral hematocrit and plasma T-1824 concentration suggests that the whole body-peripheral hematocrit ratio is unaffected by anesthesia, and that the dilatation or contraction of viscera containing a high concentration of erythrocytes is not important in producing blood volume changes during general anesthesia in man.

In contrast to this, general anesthesia in the dog is accompanied by marked changes in the peripheral hematocrit which are prevented or reduced by splenectomy (4, 5, 18), and plasma volume changes estimated from the hematocrit do not agree with those measured simultaneously by dye dilution. Splenectomy brings the calculations into agreement (3, 5).

Apparently the anesthetics studied do not affect the rate of disappearance of T-1824 from the plasma, since estimation of the plasma volume change by hematocrit and dye dilution agree for some time after the induction of anesthesia. The progressive divergence between plasma volume changes calculated by the two methods which begins eighty minutes after the injection of dye may be related to the fact that the rate of dye disappearance from the plasma has been found to diminish abruptly at this time (19).

If changes in systemic arterial and venous pressures exert directionally similar effects on capillary hydrostatic pressure, the plasma volume changes observed during anesthesia can be viewed as resulting from accompanying hemodynamic alterations. It is conceivable that a decrease in systemic arterial pressure may accompany an increase in mean capillary hydrostatic pressure, and *vice versa;* however, it is less likely that capillary pressure can vary oppositely from venous pressure (20). Hence, it is interesting that a significant decrease in plasma volume was observed in the present study only when venous pressure was elevated. Venous pressure changes in the arm may not equal those occurring elsewhere, but in man the intrathoracic venous and right atrial pressures appear to be elevated during cyclopropane and ether anesthesia by an amount similar to the increase in peripheral venous pressure (21–23). A limited number of observations suggests that the intrathoracic venous pressure is slightly reduced during thiopental anesthesia (24), and this again agrees with the change in peripheral venous pressure.

During deep anesthesia with cyclopropane the plasma volume may also be reduced by an increase in the general level of venous pressure resulting from intermittent positive pressure inflation of the lungs, and by an increase in mean arterial pressure. Increased arterial pressure has been noted previously during cyclopropane anesthesia (21, 22), but it remains unexplained.

Increased arterial pressure was observed only during the period immediately following the induction of ether anesthesia, and it occurred in several instances during demonstrable asphyxia. Venous pressure remained elevated during the "late" period of ether anesthesia but the elevation was less than half that observed shortly after induction. It is therefore possible that the decrease of plasma volume persisting into the later period of ether anesthesia resulted from an incomplete reversal of earlier changes.

The plasma volume changes during thiopental anesthesia were in the direction expected from the alterations in arterial and venous pressure. Increased plasma volume following the administration of morphine occurred in the absence of any consistent change in arterial pressure, although a decrease was noted in most instances.

The clinical significance of these observations is difficult to determine. In man the extracellular fluid volume remains essentially constant during anesthesia with cyclopropane, ether, or thiopental (25), so that one way of viewing the present results is to suppose that thiopental anesthesia and morphine result in partial depletion of the extravascular extracellular fluid volume, while cyclopropane and ether do not. Cyclopropane or ether might therefore be better tolerated by patients with a depleted extracellular fluid volume than thiopental. In support of this view it has been found that pentobarbital, whose hemodynamic effects resemble those of thiopental, reduces the rate of restoration of the plasma volume after hemorrhage in dogs, cats, and rabbits (26).

SUMMARY AND CONCLUSIONS

1. General anesthesia in man is accompanied by changes in the plasma volume which are opposite in direction to simultaneous changes in arterial and venous pressures.

2. The contraction or dilatation of viscera containing a high erythrocyte concentration is probably not important in producing changes in the circulating blood volume during general anesthesia in man.

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REAGENTS and REACTIONS 2

recent progress

For steroid hormone assay, enzymatic digestion of blood and urine often proves superior to old-fashioned acid hydrolysis. With β-glucuronidase the steroid nucleus can be split from its glucuronide conjugate: gently, specifically, accurately. At a recent Laurentian Hormone Conference, many investigators agreed on the advantages of this new method. But they also stressed the importance of using a reliable reagent. While bacterial and spleen glucuronidases may give inadequate, variable recoveries, participants from the University of Minnesota showed that "of all the glucuronidase preparations on the market," *Warner-Chilcott's Ketodase* (beef liver β-glucuronidase) was "the only fully effective preparation...."[1]

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a word of caution

The new radioactive tracers help in many research problems but not necessarily in the hospital laboratory, according to D. A. MacFadyen, (Rush Prof. of Biochemistry, U. of Ill. Coll. of Med.). Specifically, "measurement of blood volume by means of erythrocytes labeled with radioactive isotopes... offers no advantages over measurement by means of dyes, T-1824 for example."[2] *Warner-Chilcott's Evans Blue (T-1824)* matches any tracer technic in accuracy and has never shown toxicity in over 30 years of clinical experience. On the other hand, carcinogenic activity of P^{32} and protein changes in albumin made radioactive with I^{131} were recently reported.[3,4] The Evans Blue technic, "readily mastered by the average technician,"[5] is outlined in a new simplified procedure booklet (check coupon below).

1. Recent Progress in Hormone Research, New York, Academic Press, Inc., 1954, vol. 9, pp. 44, 411, 440. 2. Yearbook of Pathology and Clinical Pathology, Chicago, The Year Book Publishers, Inc., 1953-1954, p. 262. 3. J. Clin. Investigation *32*:246 (Aug.) 1953. Ibid. *33*:136 (Feb.) 1954. 4. Proc. Soc. Exper. Biol. & Med. *86*:266 (June) 1954. 5. Surg., Gynec. & Obst. *97*:709 (Dec.) 1953.

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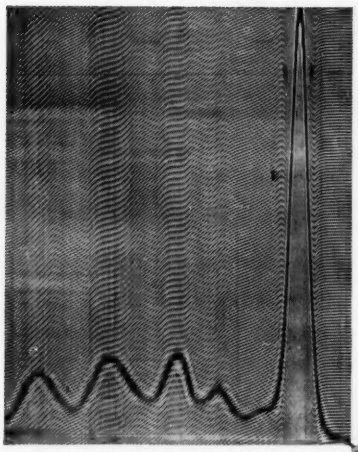
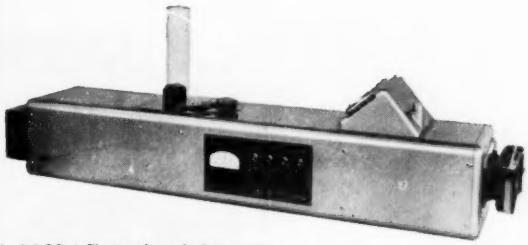


Ascending branch of albumin peak.



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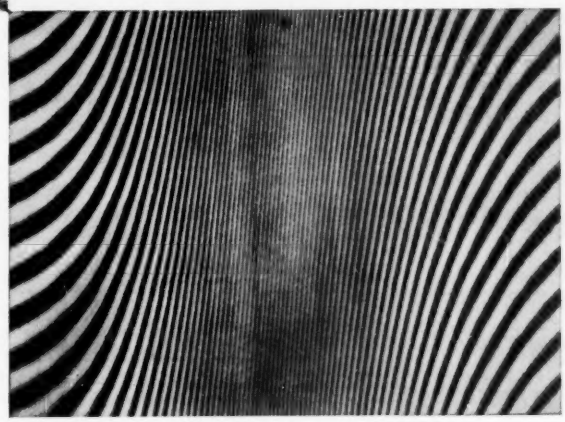

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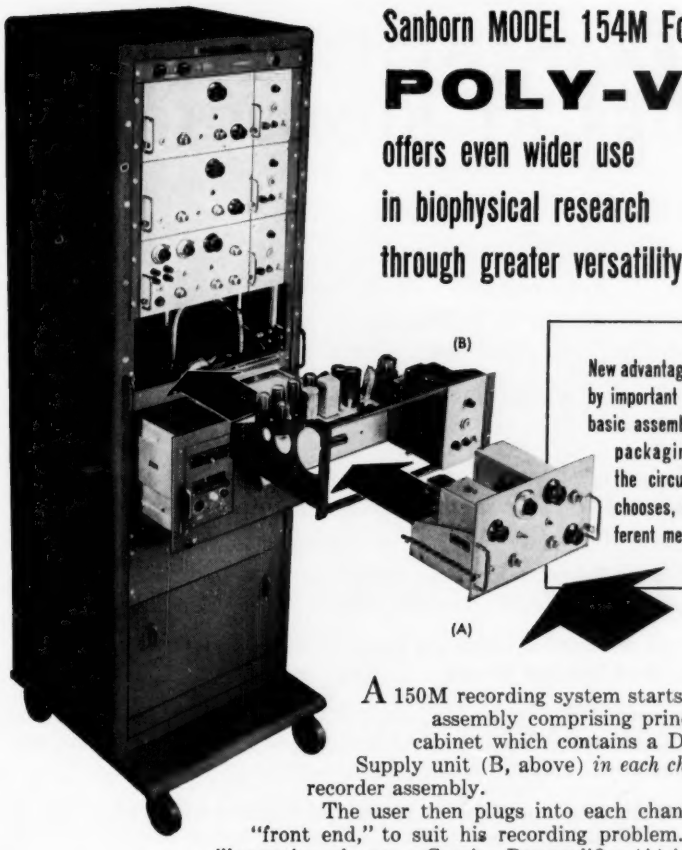

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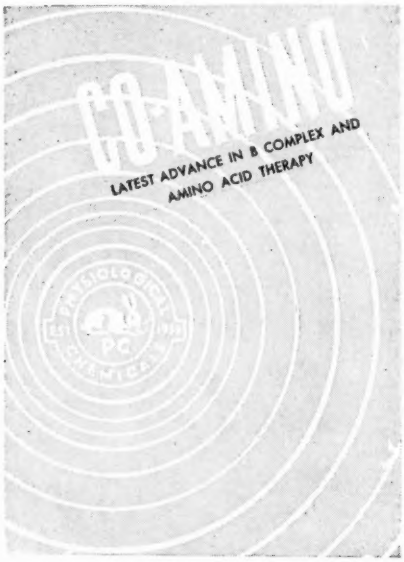

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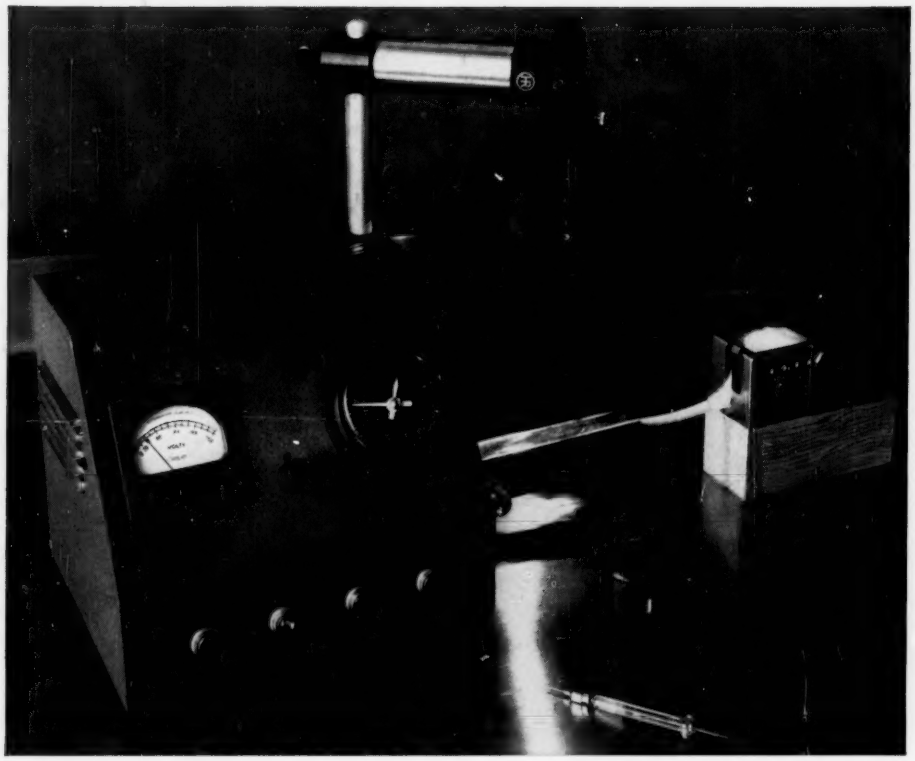

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